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"Ecology and genetics of Mnemiopsis leidyi A. Agassiz 1865: a field study on the invasive ctenophore in North Europe"

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“My kids will tell their children: eat your jellyfish!”
Daniel Pauly

ABSTRACT

Mnemiopsis leidyi A. Agassiz 1865 is an American ctenophore that has invaded European waters in the last decades. The reliable, but still debated, relationship between this invasion and the collapse of the stocks of anchovy *Engraulis encrasicolus* (Linnaeus, 1758) in the Black Sea has concerned managers and scientists because of the possible damages on the fish stocks in the new invaded areas, involving several scientists in researches about this combjelly. The lack of data about Ctenophora has hampered this research effort in the past years, but important results have been reached. The pattern of invasion has been clarified, with the identification of two subclusters (one in North Europe and a second one in Mediterranean, Black and Caspian Sea) arising from two invasions of individuals coming from different native populations along American coasts. Moreover, publications of the complete sequences of nucleic and mitochondrial DNA have made this species the model organism for genetics in its Phylum.

In this study *M. leidyi* is analyzed with two different approaches. At first an ecological descriptive survey is carried out in Denmark in the Aarhus Bay where water masses from Baltic Sea and North Sea overlap and mix each other. The presence of two populations with different size distributions within this stratified water column is showed. Also the abundance variations are related with biotic (zooplankton abundance) and abiotic (temperature) factors to explain the pattern.

The second approach involves analysis carried out in Belgium with ten DNA markers. These have been used for *M. leidyi* In particular for population genetics and for molecular identification, since the presence of the morphologically similar *Bolinopsis infundibulum* that can be misidentified. ITS region is the marker used to point out the species, while seven microsatellites give data for population genetics and to be compared with previous studies. Also new primers for cytb and cox1, markers on mtDNA, are successfully created to investigate the presence of SNPs.

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1 INTRODUCTION

Invasive species have become in the last decades a worldwide problem that concerns scientists, managers and public opinion with increasing interest. The number of species that have invaded new ecosystems have grown in the last decades faster than in the past age and new areas are colonized every year by non-indigenous organisms (Streftaris et al., 2005; Wonham and Carlton 2005; Ricciardi 2006; Leuven et al. 2009) Public opinion and managers have usually a reduced perception of the real entity of this problem, just aware of the events that are (or could be) out of control affecting endemic species and ecosystems recognized as symbols or hampering major human activities as tourism or big factories. On the other hand scientists, thanks to their knowledge about the ecologic dynamics and typical species of ecosystems, are expected to have a better overall idea about this issue, for example taking in account also those species that because of a reduced biomass or typical characteristics or short permanence in the new ecosystem are unknown to managers and public.

A researcher can approach the problem of invasive species from different points of view. The concerns mentioned above about the damages to ecosystems and human activities can push toward applied researches for solutions and preventive actions in order to limit the negative effects of the invasive species reducing their abundance or avoiding them from spreading in new habitats. Nevertheless, more purely theoretic topics can be addressed studying the non-indigenous species. Phenomena as adaptation and microevolution on a short timescale have the possibility to be studied without the laboratory constrictions maybe on species previously considered of low interest that acquire new attraction and can lead to new discoveries in phylogenetics, population genetics, ecology, behavior biology, and resolving issues otherwise difficult or impossible to deal with.

Particular interest has aroused for the increase of blooms of invasive jellyfish (Shiganova, 1998; Graham, 2001; Mills, 2001; Link and Ford, 2006; Lynam 2006). Several species as giant Nomura *Nemopilema nomurai, Phyllorhiza punctata, Pelagia noctiluca, Rhopilema nomadic, Aurelia spp., Cassiopea andromeda* (Graham et al., 2003; Bayha, 2005; Graham and Bayha, 2007; Boero and Shiganova, 2009; Richardson, 2009; Ozturk and Isinibilir, 2010) colonized in the last years regions out of the native range all over the world. Richardson, 2009, treats these invasions along with the higher abundance and the higher frequency of blooms of some endemic species. These problems are joined due to common features that can be sorted out analyzing both their causes and consequences. Climate change is one of the factors whose influence has been claimed as one of the most important causes in altering the jellyplankton equilibrium. Raising of temperature, in fact, can extend geographically and temporally the ecological niche of these species, while the stratification of the column due to the higher temperatures can shift trophic webs from diatoms-based to flagellates-based (Cushing, 1989) promoting jellyfish instead of bony fishes (Parson and Lalli, 2002) leading to greater blooms and favoring new species. Naturally also humans are not only victims as usually considered by managers and public opinion, directly supporting the presence and the permanence of non-indigenous jellies and altering the balance of native jellyplankton life cycles. The intensification of rapid large-scale transport by ships and the little attention paid to the treatment of ballast water and fouling led several species to be carried away from native habitat to new suitable regions as polipoids, ephirae and medusoid stage (Gollasch, 2006, 2007; Graham and Bayha,2007; Nentwig, 2007). Overfishing is another possible explanation to these phenomena since the collapse of fish stocks decrease the pressure on jellies of predation and competition, making more food items available and setting empty ecological niches where the jellysifh can easily take over (Elton 1958, Hufbauer and Torchin, 2007). Also pollution events that bring to eutrophication and hypoxia events destabilize ecosystems and shift the community towards new invasive species that can survive thanks to peculiar characterstics as the tolerance to low oxygen concentration (Decker at al., 2004).

Among invasive species, *Mnemiopsis leidyi* A. Agassiz 1865 is one of the most dangerous and known. *M. leidyi* belong to the phylum Ctenophora, order Lobata and its native range runs along the American East Coast. It is part of the natural zooplankton of the Atlantic coast from Massachusetts to Southern Argentina, either in coastal or estuarine areas (Kremer, 1994; GESAMP, 1997). The great extension of this area, along with a certain morphologic plasticity, led in the past to problem of misidentification. *Mnemiopsis leidyi, M. gardeni* and *M. mccradyi* are just the latest names used to describe the individuals from the species afterwards recognized as *Mnemiopsis leidyi* (Harbison and Volovik, 1994; Seravin, 1994a, b) as confirmed by genetic analysis (Bayha, 2005). Bayha (2005) also showed a population highly structured along the American coasts. Population of South America is well defined with the presence of endemic alleles of the markers analyzed, while in North America the population is divided in subpopulations with a particular geographical pattern near Cape Hatteras that seems to divide a northern and southern region in the US waters.

The first observation of *M. leidyi* in European waters dates back to the early 1980s in the Black Sea, when the species was described as *Mnemiopsis mccradyi* (Zaika and Sergeyeva, 1990). In the late years of that decade first blooms occurred along with the collapse of *Engraulis encrasicolus* (Linnaeus, 1758) (Kideys 2002, Knowler 2005), even if the relation of this fishery drop with combjelly bursts is still debated (Bilio and Niermann, 2004). *M.leidyi* probably was carried over the Black Sea in ballast waters of commercial ships (Shiganova et al. 2001, Vinogradov et al., 1989) and in few years has successfully spread in new areas. It was detected in the Azov Sea in 1988 (Studenikina, 1991), in different localities of the Aegean Sea since 1990

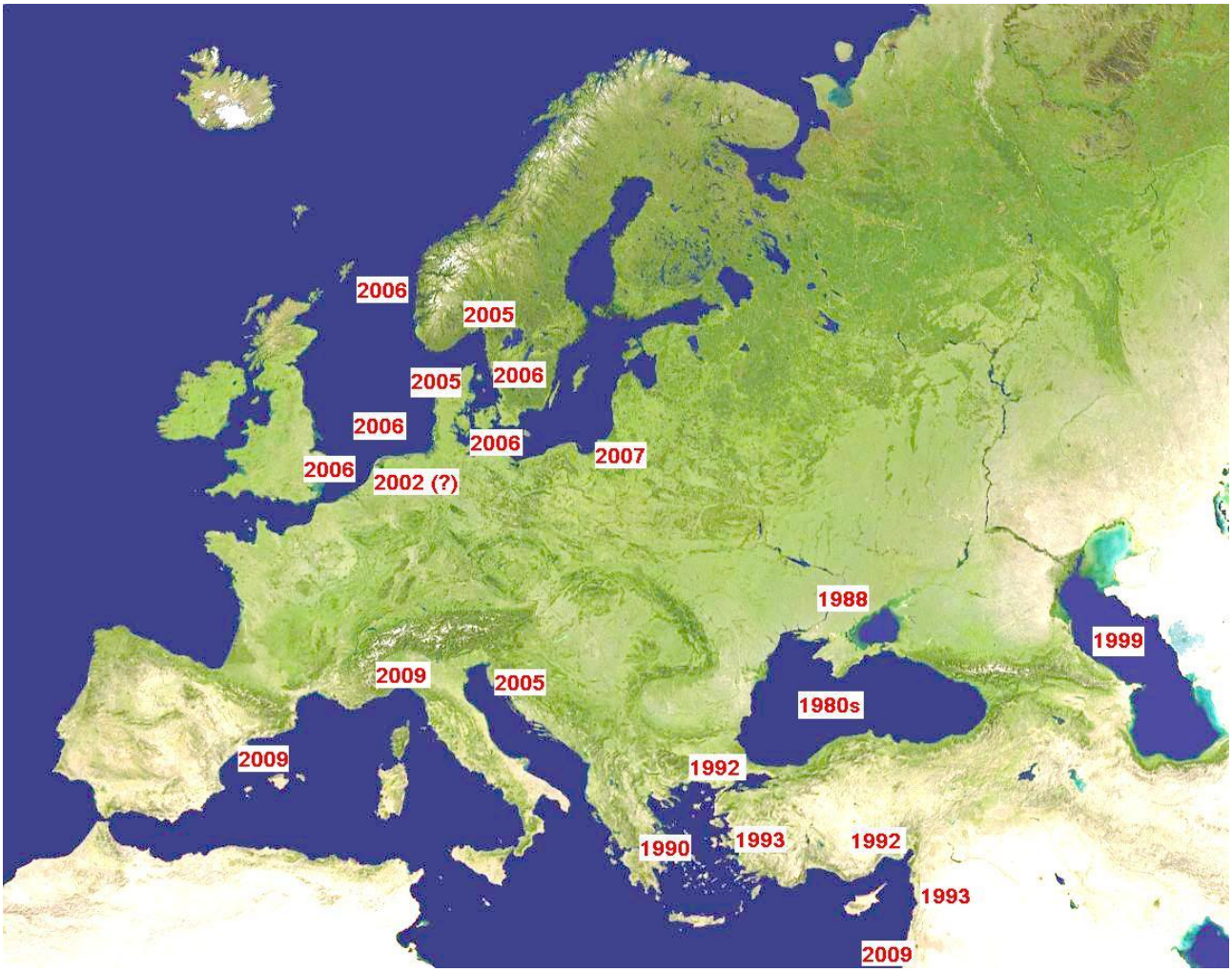


Fig. 1.1 Map of Europe with years of first observations of *Mnemiopsis leidyi*

(Kideys and Niermann, 1994; Shiganova et al., 2001), in the Marmara Sea in 1992 (Shiganova, 1993) and in Caspian Sea in 1999 (Ivanov et al., 2000). Its presence in the Mediterranean Sea was reported in the Aegean basin, along the Syrian coasts in 1993 (Shiganova, 1997), but the real entity of this invasion has been recognized just in the last years when *M.leidyi* has been observed in the Gulf of Trieste in 2005 (Shiganova and Malej, 2009) and along Tyrrhenian Italian (Boero and shiganova, 2009), Spanish (Fuentes et al., 2009) and Israeli coasts (Galil et al., 2009) in 2009. Meantime a second invasion was going on along the north coasts of Europe. First observations *Mnemiopsis leidyi* were in fact recorded in 2005 in the Oslofjorde (Oliveira, 2007) then in 2006 in different localities in the South Baltic along German (Javidpour et al., 2006; Kube et al., 2007), Swedish (Hannson, 2006) and Danish coasts (Tendal et al., 2006), but also near Bergen, on the Norwegian north coast (Hannson, 2006), and along the North Sea coasts near Helgoland in Germany (Boersma et al., 2007), Zeebrugge in Belgium (Dumoulin, 2007) and Wadden Sea and the Scheldt estuary in The Netherlands (Faasse and Bayha, 2006) and in 2007 in Polish South Baltic (Janas and Zgrundo, 2007). Genetic analysis carried out with different nuclear DNA markers to compare populations from different invaded areas and individuals from the native American coasts showed that these two invasion pathways must be considered as independent events. Two distinct clusters may in fact be detected in Europe, a southern one (Ponto-Caspian Region) and a northern one (Baltic and North Sea), reflecting the different origin of these populations. The southern cluster seems in fact related with native population in the Gulf of Mexico (Florida coasts), while northern one with populations living northward in the American Atlantic coasts, off New England (Ghabooli et al., 2010; Reusch et al., 2010). In this scenario it seems reasonable to see the invasion of the Caspian and Mediterranean Seas as secondary events, with individuals from the Black Sea translocated once again with ballast waters to further areas (Shiganova et al., 2004, 2005, Galil et al., 2009; Richardson et al., 2009; Shiganova and Malej, 2009).

Specific characteristics underlie the great success of this invasion. *Mnemiopsis leidyi* is a eurytherm and euryhaline species (Kremer, 1994; GESAMP, 1997) and can tolerate low oxygen concentrations, being able to survive in much deteriorated habitats no longer suitable for competitors like bony fishes (Decker at al., 2004). Also the diet of this species is generalist, allowing the exploitation of several and different food sources as crustacean zooplankters (Kremer, 1979; Feigenbaum and Kelly, 1984; Purcell et al., 1994; Purcell and Decker, 2005) and early stages (eggs, larvae) of shellfish and fishes, also of species economically exploited (Purcell et al, 1991; Purcell et al, 1994b) according with the “niche breadth-invasion success” theory of Vasquez (2006). Furthermore, *M. leidyi* can show very sharp increase in abundance in short time, outbreaking with great blooms soon after food items ones (Feigenbaum and Kelly, 1984; Kremer, 1994). This is due to the peculiar reproduction of this species that is able to self-fertilization (Harbison and Miller, 1986) as a simultaneous hermaphrodite (Mayer, 1912) and to reproduce at a larval stage few days after the hatching (Martindale, 1987) producing thousands of eggs with low energy costs (Reeve et al., 1989). Another reason explaining the success of *M.leidyi* could be found in the absence of predators in some of the new ecosystems. Evidences of this could be found in the drop of abundance of this ctenophore in the Black Sea after the arrival of another non-indigenous jelly, *Beroe ovata*, a known predator of *Mnemiopsis leidyi* (Purcell et al., 2001; Kideys, 2002; Bilio and Niermann, 2004) and in the intraguild predation showed by Hosia and Titelman (2011) with *Cyanea capillata* and with *Beroe gracilis* (2011b) that could hamper the spreading and the success of M. leidyi in the invaded North Europe region.

Two kinds of experiments were carried out in this study. The first descriptive ecological work was aimed to give info about *M. leidyi* abundance, distribution and how they are influenced by biotic and non-biotic factors. These aspects are the first to be investigated for an invasive species to understand which part of the ecosystem is affected and which endemic species can be damaged. Moreover, these data are useful in planning sampling surveys or actions to limit the species itself and also as input to model correctly the movement of the species in order to identify new areas where a suitable habitat can allow the settlement

of self-sustainable populatins, which of these areas are likely to be reached by the species and to try to prevent these events with effective countermeasures. The second part was a genetic analysis aimed in identify and using several markers, targeted sequence obtainable amplifying DNA with certain primers with an optimized PCR protocol. Thanks to the variability among individuals (differences in length or substitutions of bases in certain positions), a marker (or, better, a set of markers) can allow a comparison among sequences obtained from different individuals. In this way each sample can be characterized and assigned to a certain group, population or taxa, providing information for studies of phylogenetics, population dynamics, ecology and taxonomy.

The first part of this study was carried out at the University of Aarhus during October, November and December 2010 and focused on *M.leidyi* population in the Aarhus Bay, Denmark. The Bay faces the South Kattegat and receives superficial waters from the Baltic area (water passed through the Danish Straits) and deep water coming from the north (from North Sea passing through the Skagerrak). The presence of these two inflows makes possible the presence in this area of two different populations, water coming from basins where the ctenophore has already been collected. A first pilot study was needed to decide the best sampling method, since it is still missing a standardized protocol. After, analyses focused on the biovolumes and the numbers of individuals caught at different depths. Comparing these values and relying also on the differences in size distribution among layers of the column, differences among two eventual populations belonging to the different water masses were tried to be found. Survey about the possible factors influencing the abundance and distribution of *M.leidyi* were also carried out. Samples of zooplankton were caught thanks to a pump and taxonomical identification was carried out. Calculation of abundance of some of the most important of these taxa (or functional groups taxonomically unknown as nauplii) was also used to try to identify possible prey items of this ctenophore, whose diet composition in the invaded areas is still not totally understood. Also the most important oceanographycal factors were analyzed to describe the environment experimented by this organisms and the possible limits that they have to cope with. At last the presence of potential predators (*Cynea, Beroe*) (Boersma et al, 2007; Hosia and Titelman, 2010) was noted to obtain some indirect evidences of some actual relationship among jellyplankton species..

The second part of this study has been carried out in Oostende, Belgium, at the Animal Science Unit – Fisheries of ILVO, the Institute for Agricultural and Fisheries Research and lasted 4 months (September 2011- December 2011) and was focused on genetic analyses on *Mnemiopsis leidyi* within the project MEMO. Samples caught over the whole area of interest of the project (North Sea and The Channel) were sent to Oostende, where crude DNA was extracted and analyzed and up to ten markers were analyzed.

The first marker used in this study is the ITS region, a non-coding DNA region in the transcriptome of rRNAs. ITS was used as molecular identification of Mnemiopsis leidyi as in previous studies (Bayha, 2005; Faassee and Bayha, 2006; Fuentes, 2010), a test shown to be needed because of fragility of these animals, easily destroyed during capture and rapidly degraded under fixative, and also because of misidentification between similar species (Faasse and Bahya, 2006) or overestimation of the genetic divergence in the same species with high morphological variability (Dawson, 2005).

A second set of markers was considered after Reusch et al., 2010, a paper where thanks to different alleles frequencies of seven microsatellites in individuals obtained from a worldwide sampling it is argued the presence of two different invasions of M. leidyi in Europe resulting in a northern and a southern cluster. The same seven microsatellites were used to screen the individuals of the project MEMO and the ones from Italy and to detect eventual subclusters and to gather data to be compared with the ones from previous studies.

For the last two markers used in this study the mitochondrial DNA was taken in account, a molecule already used for different species as a genetic marker for phylogentics, population genetics and biogeography (Avise, 2004). The cytochrome b (Cyt b) is an important marker used already for several species (e.g.

Montegelard et al., 1997; Parson et al., 2000; Castresana, 2001). It has already been useful in the studies about M.leidyi as molecular marker for mtDNA when it has been impossible to amplify the region coding for the cytochrome oxidase 1 (Cox1). This last gene, in fact, is one of the most important markers used for animals since it is easy to amplify and a big database has been created to do molecular identification of several species out of it. Cox1 has also been used for population genetics on jellyplankton species (Holland et al., 2004). The goal in both these last markers was to identify SNPs, positions in the sequence where two bases can appear. They are useful in population genetics since it is assumed that in every population the two bases allowed for the SNP have different frequencies. Genotyping individuals and

Eventually, this study is a resume about the lab and field work about the invasion of *Mnemiopsis leidyi* in Europe, with a particular focus on the situation in North Europe. The main goal is to optimize protocols for ecological and genetic studies showing the practical possibility to gather data to work on.

2 MATERIAL AND METHODS

2.1 Denmark – 2010 – ecological study

2.1.1 Pilot study

The 15th October 2010 a survey was done to demonstrate the actual presence of individuals of *Mnemiopsis leidyi* in the Aarhus bay trying out the validity of the sampling protocol too. The area surveyed was the same that would have been visited later for the proper study (see AREA OF STUDY). The CTD data showed 3 layers in the water column. A depth within each layer (4m, 11m and 25m) was chosen to collect jellyfish and zooplankton. Jellyfish were collected with a net (the same used in the following experiment) hauled horizontally along transects for 10 minutes. One haul was done for every depth. A flowmeter was attached at the net aperture to calculate the filtered volume. Three samples of zooplankton were also collected at every depth with the pump used in the experiment. Samples from this pilot study were useful to demonstrate the presence in the bay of *Mnemiopsis leidyi* and to make a previous taxonomical analysis of the zooplankton. However, the data about the volume and the number of caught combjellies were not included in the statistical analysis because of the different method used in the pilot study (horizontal hauling by transects) and in the proper experiment (vertical hauls). Transects turned out to be too time consuming leading to few replicates and also gave problems with calculation of the densities because of difficulties and lack of precision in measuring the volume of water filtered in the upper layers while pulling up the net from the deeper layer since the net was not closable.

2.1.2 Area of study and hydrography

The sampling site was situated in one of the deepest places in the Aarhus Bay near the peninsula of Helgenæs by the Sletterhage lighthouse. The bottom depth varied between 30m and 45m. The area was chosen because of the high probability to find stratification in the water column.

At every sampling event the ship was anchored and the CTD data were recorded first. The CTD recorded temperature (°C), salinity (°/oo), oxygen (%) and chlorophyll-a fluorescence (ug/l). The CTD was dropped only once every sampling day assuming the column stratification system not changing in the few hours of sampling.

2.1.3 Jellyplankton collection

A ring trawl (length 3m, aperture 1m in diameter, mesh 1mm) was used to collect the jelly plankton. In case of water column stratification the net was dropped until the ring reached the lowest depth of the deepest layer and then pulled up making a vertical haul .This process was repeated 5 times. After that, the same protocol was used to survey the superficial layer, pulling the net from the bottom of the upper layer to the water surface for 5 times. With a 3 layered system, only 3 hauls were done for the whole water column, while 5 samples were taken from the bottom of the intermediate layer up to the surface and another 5 starting from the bottom of the most superficial layer to the surface.

Because of the fragility of the combjellies, the individuals caught were stocked in different buckets with a mark to be recognized and analyzed few hours after the sampling. No fixatives were used.

2.1.4 Jellyplankton analysis

In the lab, proceeding one bucket at time, the jellyplankton was poured in a lab plastic tray and each individual measured with the accuracy of 1mm. The length (mm) was measured from the aboral ending of the umbrella to the end of the lobes. The individuals were then put on a 1mm mesh to get rid of excess

water and then into a measuring beaker to calculate the biovolume (ml). During the second survey 2 samples out of 5 from the superficial layer, 2 samples out of 5 from the intermediate and superficial and 1 sample out of 3 from the whole column were analyzed only by calculating the biovolume without measuring the length of the individuals. The buckets analyzed with the whole protocol were chosen randomly.
The presence of other jellyfish species, in particular the genera *Cyanea* and *Beroe*, was also noted. Their size was measured as the diameter of the umbrella seen from above (*Cyanea*) or as the length from the aboral ending to the oral aperture (*Beroe*).

2.1.5 Zooplankton collection and analysis

Zooplankton samples were collected on every cruise after the jellyplankton harvest. Three zooplankton samples were collected dropping the pump to a depth in the middle of each water stratum
A KC Zooplankton Pump was used to collect the samples and the water was filtered through a 63 μm mesh. The pump was activated by a remote control on board of the Tyra and every time it run for 5 minutes. Considering the nominal filtering rate 500 L/m a volume of 2500L must be considered filtered in every sampling. The filtered plankton was then poured into dark bottles where some Lugol was added to preserve the organisms from decomposition.
In the lab bottles were gently mixed to suspend the plankton precipitated at the bottom and then every sample was divided 4 or 5 times with a zooplankton splitter according to the density of the sample itself. This subsample was so analyzed under a stereoscope microscope with magnification between 2,5x and 3x. Zooplankton species were identified with manuals and keys, according also with the species previously reported in the bay. Ten classes (different taxa or different developmental stages of the same taxon) were established and the number of individuals of each class was counted to estimate the abundance.
An algae bloom occurred during the sampling period. This can have led to errors in the counting step, but also have clogged the pump net. In this study these errors are considered so slight to be ignored.

2.1.6 Data analysis

Mnemiopsis leidyi individuals were measured and divided into thirteen size classes of 5 mm (smallest class 0-5 mm; biggest class >60 mm). Total number of individuals (TN) and biovolume (BV) were recorded for every vertical haul.
If the layers in the water columns were 2, 2 data sets were obtained:

- data from the superficial layer; (A1)
- data from the whole water column. (B1)

In case of 3 layers, the data sets were 3:

- data from the superficial layer; (A2)
- data from the superficial + intermediate layers; (B2)
- data from the whole column. (C2)

In order to show the presence of individuals of this species in every layer of the column, a student T-test was carried out. For the 2-layers columns, the T-test was between the TNs (BVs) of the samples coming from the surface (A1) and the TNs (BVs) coming from the whole column (B1).For the 3-layers columns, more T-tests were carried out. The first compared the data from the surface (A2) with the TNs (BVs) coming from the surface plus the intermediate layer (B2), while a second T-test compared this latter (B2) with the TNs (BVs) from the whole column (set C2). A statistically significant output from these tests showed that fishing only in the upper layer(s) is different than fishing in the whole water column and, then, that the species is present in every one of the water masses considered.

Once detected the presence of *M.leidyi* in the 2 or more layers, the TN (BV) of individuals for the deepest layer was calculated as formula (1) for the 2 layers columns and as formula (2) for the 3 layers columns:
TN deep layer = TN(B1) – TN(A1) (1)
TN deep layer = TN(C2) – TN(B2) (1)
The number of individuals caught in the intermediate layer of the 3-layers columns was calculated as formula (3):
TN intermediate layer = TN(B2) – TN(A2) (3)
The same was done for the BV.
The TN and the BV calculated for every layer were used to calculate the density of individuals and the density of biovolume as (4):
TN (or BV) DENSITY = TN (or BV) / (0,5m * 0,5m * LAYER THICKNESS * 3,14)
From 3 to 5 replicates of BV and TN densities (respectively ml/l and individuals/l) were obtained for each water mass surveyed. A 2 tailed t-test was used to compare the layers. In case of statistically significant output, the populations in the two layers compared have different density (number of individuals and/or biovolume)
A finest work was done on the 13 size classes. For every sample the number of individuals of each class in the superficial layer was obtained just counting. Analyzing one class at time, the mean number of individuals from replicates in the surface was subtracted from the number of individuals caught in the whole water column. This subtraction was done one class at time subtracting to the number of individuals of that class from the whole column the mean value of the individuals found in the superficial replicates of that sampling day. In this way 5 replicates of the number of individuals in the deep layer were obtained. On the basis of this class division, the cumulative number of individuals was calculated for each layer and the size distributions of different strata were compared with a Kolmogorov-Smirnov test, using the number of classes (13) as the size of samples. Densities of every class were measured and the same class was compared among layers of the same survey with a t-test.
The zooplankton samples were splitted 4 or 5 times and the number of individuals for every class was counted. The number of individuals (once multiplied by the dilution factor) was then divided by 2500 (volume in liters of water filtered by the pump). In this way 3 replicates of the density (individuals/liter) for every class were obtained for every layer surveyed. The average of these values was considered the density of that class in the layer. A 2 tails T-test was then used to compare the same class in different layers.

2.2 Belgium – 2011 – genetic study

2.2.1 MEMO Interreg

ILVO is a Flemish Scientific Institute belonging to the Flemish Government's Agriculture and Fisheries Policy Area. ILVO is the leader institute of the MEMO Iterreg (**M**nemiopsis leidyi **E**cology, **M**odelling and **O**bservation), a project involving five research partners (ILVO, IFREMER, ULCO-LOG, CEFAS and Deltares) across Belgium, France, Netherlands and England meant to coordinate and finance an overall research effort on *Mnemiopsis leidyi* in The Channel and North Sea. ILVO is the institute in charge of genetic analyses on samples caught for MEMO.

2.2.2 Samples and DNA extraction

Specimens of ctenophores were caught in The Channel and in the North Sea (both coastal and offshore water). These samples were not collected following a sampling campaign arranged a priori, but were the result of several oceanographic surveys and occasional catches ranging from August 2010 to October 2011. Sampling methods varied from nets hauls to manual catching. Individuals caught were kept in jars filled

with ethanol and sent to ILVO institute in Oostende for genetic analyses. Also two individuals morphologically identifiable as *Mnemiopsis leidyi* were caught on Italian waters off of Baia Blu in Lerici (SP) (44° 04' 57.26" N, 9° 53' 13.31" E) on 17/10/2011, preserved in ethanol and sent to Oostende to carry out molecular identification.

DNA EXTRACTION PROTOCOL

- Put 100mg of animal tissue in a microcentrifuge vial
- Add 120µl of EDTA 0.5M solution, ph 8.0 and 500µl of Nuclei Lysis Solution. Vortex well
- Add 17.5µl of Proteinase K solution (20mg/ml). Vortex well
- Incubate at 55°C overnight or at 55°C for 3 hours and in this case vortex every hour.
- Add 3µl of RNase A solution (4mg/ml) in the nuclear lysate. Mix gently the microcentrifuge vial by turning 5 times
- Incubate 30 minutes at 37°C
- Let cool down the samples at room temperature for 5 minutes
- Add 200µl of Protein Precipitation Solution. Vortex at maximum speed for 20 seconds
- Centrifuge at 14000 tpm for 10 minutes. The precipitated proteins are evident as a white pellet
- Poor the supernatant in a new microcentrifuge vial
- Add 600µl of icy isopropanol
- Mix gently the microcentrifuge vial by repeatedly turning
- Keep the microcentrifuge vial at -20°C for at least 1 hour
- Centrifuge at 14000 tpm for 10 minutes
- Get the supernatant off. The DNA is evident as a white pellet
- Add 600µl of ethanol 70%
- Rinse the pellet and mix gently the microcentrifuge vial by repeatedly turning
- Centrifuge at 14000 tpm for 10 minutes
- Get the supernatant off
- Put the microcentrifuge vial upside down on blotting paper and leave the microcentrifuge vial open to let it dry at air
- Add 100µl of TE-buffer 1X. Pipette slowly to make the pellet solve
- Incubate at 65°C for 1 hour or at room temperature overnight
- Keep the DNA at -80°C

Reagents

- Nuclei Lysis Solution (Promega, ref: A7943)
- Protein Precipitation Solution (Promega, ref: A7953)
- Tris Base (Promega, ref: A5135)
- EDTA-Na_2 dihydrate (BDH, ref: 443885J)
- NaOH p.a. (BDH, ref: 102525P)
- HCl 37% (Merck, ref: 1.00317.2501)
- Proteinase K, recombinant PCR grade lyophilizate (Roche, ref: 3115879)
- Rnase A (Roche, ref: 10109169001)
- Isopropanol p.a. (Serva, ref: SERA39559.02)
- Ethanol p.a. (Merck, ref: 8.18760.2500)
- 18.2 MΩcm water (Arium, Sartorius)

Box 2.1 Promega (Belgium) DNA precipitation protocol used to extract crude DNA.

Samples arrived at ILVO underwent the crude DNA extraction process. It is a Promega protocol based on DNA precipitation (box 2.1).

2.2.3 ITS

The ITS (Internal Transcribed Spacer) region is located in the transcription unit (45S) that encodes the rRNAs 18S, 5S and 28S and includes 2 regions (ITS-1 and ITS-2) flanking the 5,8S rDna. (fig 2.1).

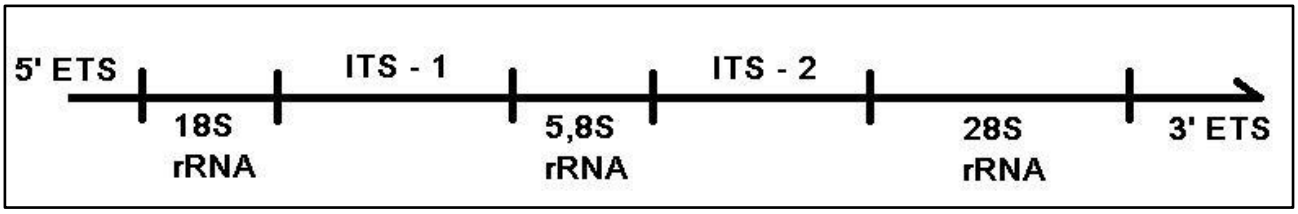


Fig. 2.1 Genetic organization in the 45S rDNA transcriptome.

ITS 1 and 2 are non-functional regions that are excised from the rRNA in the maturation process.ITS region has too many repeated sequences to be used in phylogenetic studies for taxa broader than family, but it is useful to discriminate species and it has been already used as marker for molecular identification of *Mnemiopsis leidyi* (Bayha, 2005; Faassee and Bayha, 2006; Fuentes, 2010).

The primers used in this study are KMBN-8 (ATTACGTCCCTGCCCTTTGTA) and KMBN-9 (GCAATCCCAAACAGTCCGACTCTTC) from Bayha (2005). The PCR solution included 20µl of MasterMix, 4µl of KMBN-8 work solution [5µM], 4µl of KMBN-9 work solution [5µM], 2µl DNA template and 10µl of water. The PCR protocol consists of a denaturing step (95 °C for 10 min), 36 amplification cycles (95 °C for 45 sec – 52 °C for 1 min – 72 °C for 1:30 min) and a final elongation step (72 °C for 10 min) (Fuentes et al., 2010). Amplifications were tested on a 1% agarose gel. Some samples were not successfully amplified at first instance, due to the high impurity concentration in the DNA template. DNA was then either treated with a Invitek purification kit or diluted with water to 1/100 (99µl of water + 1µl of DNA) and 1/1000 (999µl of water + 1µl of DNA) of the original concentration. After the first testes, the dilution 1/100 turned out to be the one working the best and it was used to analyze all the last samples and to amplify again the DNA of the samples that had failed in the previous PCRs.

The same Invitek purification kit was used to prepare PCR products to sequencing. This last step was performed at VIB Genetic Service Facility (GSF) at the University of Antwerp using the primer KMBN-11 (ATTTGAGCTGTTCCCTGTTCGT).

Sequences obtained from VIB were submitted on a nucleotide BLAST query on NCBI databases to be compared with the ones already deposited for the molecular identification. The species whose sequences in NCBI matched the best the one used as input was considered the species the new sequence belonged to.

2.2.4 Microsatellites

Microsatellites are sequences of non-coding DNA scattered all over the genome. They are defined as sequences where a short motif of 2-3 bases (e.g. GCA or TC) is repeated in tandem several times (Litt and Luty, 1989). Different alleles in these loci are distinguished by the number of repetitions of the short sequence (eg. $(GCA)_{10}$ and $(GCA)_{15}$) resulting in alleles discriminated by different length. Designing primers annealing with the regions flanking the microsatellite, it is possible to amplify the microsatellite and to genotype every individual, scoring each sequence of different length as an allele.

Primers used to amplify seven microsatellites in *M.leidyi* genome were considered after Reusch, 2010. Forward primers were fluorescently labeled with 3 different dyes and the primers were sorted in 2 pools. Only the primers of one pool were put in each PCR vial.

The protocol has undergone a process of optimization. For every microsatellite a work solution was obtained diluting 10µl of forward primer [100µM] and 10µl of reverse primer [100µM] in 80µl of water.
The first step was checking whether the primers worked singly. As first, 3 microsatellites (L15, L116, L211 from Pool 2) were tested with two dilution factors of 1/100 and 1/1000 over the original DNA extracted.

The PCR solution was composed of 10µl of MasterMix, 1µl of DNA template, 8µl of water and 1µl of the work solution of one microsatellite at time. The PCR protocol consists of a denaturing step (95 °C for 3 min), 35 amplification cycles (95 °C for 30 sec – annealing temperature °C for 30 sec – 72 °C for 45 sec) and a final elongation step (72 °C for 7 min). Other 3 microsatellites (L12, L13, L45.1 from Pool 1) were tested lately only on DNA diluted 1/100. The annealing temperatures used were 61°C for Pool 2 and 60°C for Pool 1. The seventh microsatellite (C1583) was added later and the single-microsatellite test was not done before the multiplex PCR.The first six microsatellites (L12, L13, L15, L45.1, L116 and L211) were tested together in a multiplex PCR. The solution in each vial consisted of 10µl of MasterMix, 2µl of DNA template, 5µl of water and 3µl in total of work solutions of microsatellites of one pool (1µl of L12, 1µl of L13 and 1µl of L45.1 in vials for Pool 1 and 1µl of L15, 1µl of L116 and 1µl of L211 in vials for Pool2). Annealing temperature was set at 60°C for both the pools. PCR products were analyzed on a 1% agarose gel.

Pool	Locus	Primers	Dye
Pool 1	L 12	(for) TTGCAATTTATGAGGGAGGTG (rev) GACTCGATCAGGATTCTTTGAG	6-FAM
	L 13	(for) CGCTTGCCTCACTTTTTAGC (rev) GCATCAAAGCAGTGCAAAATAC	NED
	L 45.1	(for) TGTCGCAAATCTGGCTGTAG (rev)CGGGGGATATTCCACAGTTC	PET
	C1583	(for) GAAACCTTGGAACCCTGGAG (rev) TCTTATGGGAGAAGATTACTTAGGC	PET
Pool 2	L 15	(for) GGTCCATGACATCGGTAAGG (rev) TGTAAATAATAAGTGCGCGACAG	NED
	L 116	(for) GAGTTTGGCGCTAAATGGAC (rev) AACACGAGCCTAACCCACAC	6-FAM
	L 211	(for) TACCCAGGCAAGCAAACTCT (rev) TCCCGGTCAGGTATATACGAA	PET

Table 2.1 Microsatellites used after Reush et al. (2010). Loci are divided in 2 pools and for each marker primers sequences and dye used in the multiplex PCR are referre.

The fluorescent signals of the PCR products obtained so far were analyzed with ABI 3130- Avant Genetic Analyzer (Applied Biosystem) with 4 capillaries at the University of Leuven.
Once obtained the seventh microsatellite C1583, a work solution was prepared like for the other six of them. Three PCR solutions were combined for every pool to increase the amount of DNA template. For Pool 1 the first solution was 10µl of MasterMix, 3µl of DNA template, 3µl of water and 4µl of work solutions (1µl for each microsatellite). For Pool 2 solutions were almost the same, but with three work solutions instead of four. Consequently the first solution for Pool 2 was 10µl of MasterMix, 3µl of DNA template, 4µl of water and 3µl of work solutions (1µl for each microsatellite); the second solution had 5µl of DNA and 2 of water and the third had 7µl of DNA and no water in it. The PCR protocol was the same as for previous tests with the annealing temperature at 60°C. The amplification was confirmed running the products on a 1% agarose gel.

Third solutions of the last test (6µl of DNA for Pool1 and 7µl of DNA for Pool2) were used with the same PCR protocol to analyze up to 80 samples. PCR products from these last tests were analyzed with ABI 3130-Avant Genetic Analyzer (Applied Biosystem) with 16 capillaries at the ILVO institute of Merelbeke.
File obtained from Merelbeke were analyzed with the software ABI GeneMapper 4.0 (Applied Biosystem). The software showed in a graph (one for each pool within a sample) the variation in fluorescence for each dye along with the different lengths of the DNA fragments amplified in the Multiplex PCR. Every dye was visualized with a line of different color (red for dye PET, black for NED and blue for 6-FAM). All the colors for each pool were considered one by one and the length corresponding to a clear peak in the graph line was considered as the length of an allele of the examined microsatellite. These peaks were manually scored rectifying the automatic peak-identification carried out by the software. Every dye had peaks with a different pattern. Every pattern was identified at the beginning for each color scrolling along all the samples to recognize profiles repeated in all of them. Only peaks adhering to this pattern were considered real ones. Peaks visible in the blanks were not scored. Microsatellites L45.1 and C1583 were showed in the same graph being marked with the same color (PET) in the same pool (primers of different microsatellites labeled with the same dye in the same PCR vial). The two sets of peaks of this graph were distinguished thanks to the non-overlapping length ranges of the alleles of each microsatellite. Once scored the peaks, the allele were identified and labeled with their lengths in a 3 digits code. Every sample was then genotyped and an output file was obtained showing the allele names of every microsatellite for each sample.
Dataset with genotypes was submitted to analyses with softwares GENETIX 4.05.2 (Belkhir et al. 1998), GENEPOP 4.0.6 (Rousset, 2008), FSTAT 2.9.3.2 (Goudet, 1995), STRUCTURE 2.3 (Pritchard et al., 2000a) and STATISTICA 6.0 (www.statsoft.com). The dataset contained the genotypes of 69 individuals for the seven microsatellites. A total of 10 groups were identified among the samples according with the source and the sampling time (Microsat. Pop. in APPENDIX V). In GENETIX the total number of alleles per locus, the number of alleles in each population per each locus, distances among populations according to Cavalli-Sforza and Edwards (1967), of Fis (f), Fit (F) and Fst(Θ) according to Weir & Cockerham (1984) and their standard deviation and a 2D AFC graphic were obtained. Furthermore thanks to the option Outils->Coversion->Fstat an input file for FSTAT software was obtained. This last program gave a gene diversity matrix per locus and a matrix of pairwise Fst that was created as a measure of the distance among populations. Because of 3 populations with 1 individual, only 7 groups out of 10 were considered. This matrix was used to create a non-metric MDS plot in STATISTICA with the default parameters. From GENETIX with Outils->Coversion->Genepop a file was obtained to test Hardy-Weinberg equilibrium within the populations with more than 1 individual using GENEPOP. In STRUCTURE a Parameters Set was created in which Length of Burning Period was 5000, Number of MCMC Reps after Burnin was 5000, Ancestry Model was set to use the Admixture Model, Allele Frequencies were correlated, and the Computer Probability of the data was used for estimating K (number of subclusters). Analysis (Job) was run comparing simulations with K varying from 1 to 7. Output files were analyzed with STRUCTURE HARVESTER (Earl and vonHoldt, 2011) to identify the most reliable number of K with methods from Evanno et al. (2005).

2.2.5 Cytochrome B (cytb)

Cytochrome b (Cyt b) is an important marker located in the mitochondrial DNA, a region quite variable and, for that, used in phylogentic studies. This locus encode for a transmembrane protein with an active role in the respiratory chain embedded in the complex III.
The amplification of a region of 361 bp in the gene Cyt b in the mtdNA was performed with primers KMBMT-80 (CAGGATAAATATGTAAGGGAGT) and KMBMT-116 (TGGGGTCAGATGTCATATTG) after Bayha (2005). PCR solution consisted of 10µl of MasterMix, 2µl of KMBMT-116 work solution [5µM], 2µl of

KMBMT-80 work solution [5µM], 1µl DNA template and 5µl of water. The PCR protocol included an initial denaturing step (94 °C for 2 min), 36 amplification cycles (94 °C for 45 sec - 50 °C for 1 min – 72 °C for 1:30 min) and a final elongation step (72 °C for 10 min). DNA was diluted 1/100 and, when first PCR failed, amplification was doubled with the dilution 1/1000. PCR products were checked on 1%agarose gel and sequencing reactions were performed at VIB Genetic Service Facility (GSF) at the University of Antwerp after purification of PCR products with Invitek kit. Primer used for sequencing was KMBMT-118 (TGTCATATTGAGCCTCTATAGT). Sequences obtained were aligned on BioEdit to identify SNPs.
After the publication of the mtDNA (Pett and Ryan, 2011) in GenBank (JF760210), a new reverse primer (GTATAAAAGTGAGTTAATCTGG) was designed to amplify a broader region (506 bp). This new primer was tested on OligoAnalyzer 3.1 by Integrated DNA Technologies (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx). Parameters were Target Type DNA, Oligo Conc 0,5 µM, Na^{+} Conc 50 mM, Mg^{++} Conc 1,5 mM and dNTPs Conc 0,4 mM. This new primer was used with the KMBMT-116. The annealing temperatures tested in the PCR protocol were 40.6 °C and 45 °C. In every PCR vial there were 10µl of MasterMix, 2µl of KMBMT-116 work solution [5µM], 2µl of new reverse primer work solution [5µM], 1µl DNA template and 5µl of water. PCR products were checked on 1%agarose gel, purified with Invitek kit and sequencing reactions were performed at VIB Genetic Service Facility (GSF) at the University of Antwerp.

2.2.6 Cytochrome C oxydase subunit 1 (cox1)

Cytochrome c oxidase 1 (cox1) is an important gene of the mtDNA. A region of 648 bp from this locus is used in almost all animal groups as a marker because the variation rate is fast enough to distinguish among species. Sequences are gathered in a database (http://www.barcodeoflife.org) whose purpose is to use cox1 in the same way of a barcode in a supermarket to carry out molecular identifications (Ratnasingham and Hebert, 2007).
Primers for a region of 787 bp were designed on the basis of the mtDNA sequence published by Pett and Ryan, 2011 (GenBank acc. number JF760210). Three primers forward and three primers reverse were designed and the combination resulting in the longest strand was chosen (frw: TTTTTTTTTCTATAATTATGGG ; rvs: CCGAAGTAAACATATGATGGGC). These two primers were tested on OligoAnalyzer 3.1 by Integrated DNA Technologies (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx). Parameters were Target Type DNA, Oligo Conc 0,5 µM, Na^{+} Conc 50 mM, Mg^{++} Conc 1,5 mM and dNTPs Conc 0,4 mM.
The first PCR tested five protocols differing in the annealing temperatures (35,2 – 40,3 – 45,9 – 51,4 – 55,4 °C). PCR solutions contained 10µl of MasterMix, 2µl forward primer work solution [5 µM], 2µl reverse primer work solution [5 µM], 5µl water and 1µl of DNA template. 2 samples were taken into account. The PCR protocol consists of a denaturing step (95 °C for 3 min), 35 amplification cycles (95 °C for 30 sec – annealing temperature for 30 sec – 72 °C for 45 sec) and a final elongation step (72 °C for 7 min). The second analysis tested twelve different protocols on 3 individuals, crossing the four working annealing temperatures with three different PCR solutions with a variable final concentration of Mg^{++}. The PCR protocol was the same as the first test but the annealing temperature of 55,4 °C was not considered. In the first solution tested there were 10µl MasterMix, 2µl forward primer work solution [5 µM], 2µl reverse primer work solution [5 µM], 5µl water and 1µl of DNA template with a final Mg^{++} concentration of 1,5mM. The second solution tested included 10µl MasterMix, 2µl forward primer work solution [5 µM], 2µl reverse primer work solution [5 µM], 4µl water, 1µl MgCl [0,5 mM] and 1µl DNA template. The final Mg^{++} concentration was 2mM. The third solution consisted of 10µl MasterMix, 2µl forward primer work solution [5 µM], 2µl reverse primer work solution [5 µM], 4µl of water, 1µl of MgCl [1 mM] and 1µl of DNA template with a Mg^{++} concentration of 2,5mM. The best annealing temperature was set at 45 °C. Another test was

done on other 8 samples testing 2 different Mg^{++} concentrations with the first and third solutions described above ([Mg^{++}] 1,5mM and 2,5mM).
Other 35 samples were tested with the annealing temperature of 45°C and the first PCR solution with a final Mg^{++} concentration of 1,5 mM.
The sequencing step was performed at the VIB Genetic Service Facility (GSF) at the University of Antwerp after purification of the PCR products with Invitek Purification Kit. The best part of each sequence was cut out with Bionumerics (ABI) and these sequences were aligned with Bioedit to identify eventual SNPs.
Another test was done considering different species, with DNA from five *Mnemiopsis leidyi* samples randomly sorted, ten *Beroe cucumis*, nine *Pleurobrachia pileus*, two *Beroe ovata* and 7 *Lovenella assimilis* . PCR protocol and solution were the same used for *M. Leidyi*. PCR results were tested on a 1% agarose

3 RESULTS

3.1 Denmark – 2010 – ecological study

3.1.1 CTD data

The CTD data were collected at arrival in the sampling area in order to detect the number and thickness of the layers in the water column.

During the first survey the water depth was 30m and 2 water masses were detected. The more superficial one went down to 16m of depth and was fresher and colder (8.17 °C and 31.97 °/oo) than the deeper one (10.64 °C and 36.03 °/oo) that went down to the bottom for 14m (fig. 3.1a). The second survey had 3 layers, a superficial one 10m thick with an average temperature of 8.01 °C and an average salinity of 32.60 °/oo , an intermediate one 18m thick with 9.52 °C as mean temperature and 34.88 °/oo as mean salinity and the deepest layer 17m thick that was the warmest (9.91°C) and most salty (35.71 °/oo) (fig. 3.1b). During the third survey the water column was divided again into 2 layers, the superficial one 11m thick that was the coldest and freshest recorded (5.88 °C and 29.36 °/oo) and the deeper one 34m thick warmer (7.01 °C) and saltier (32.12 °/oo) (fig. 3.1c).The algal bloom made it difficult to relate the fluorescence with the actual algal concentration and no results were calculated from these data. The oxygen saturation was higher in the upper layers at the two first surveys and evenly distributed at the third survey, but saturation was always higher than 57%. In order to avoid problems of contact of the net with the bottom, the frame of the net mouth was pulled up from a depth of 40m letting the net hanging deeper. In this way the inferior limit of the deep layer was considered 40m instead of 45m as recorded from the CTD. This depth would be the "sea bottom" used to measure the densities of the combjellies. Therefore the thickness of the deepest layers was 12m (second survey) and 29m (third survey).

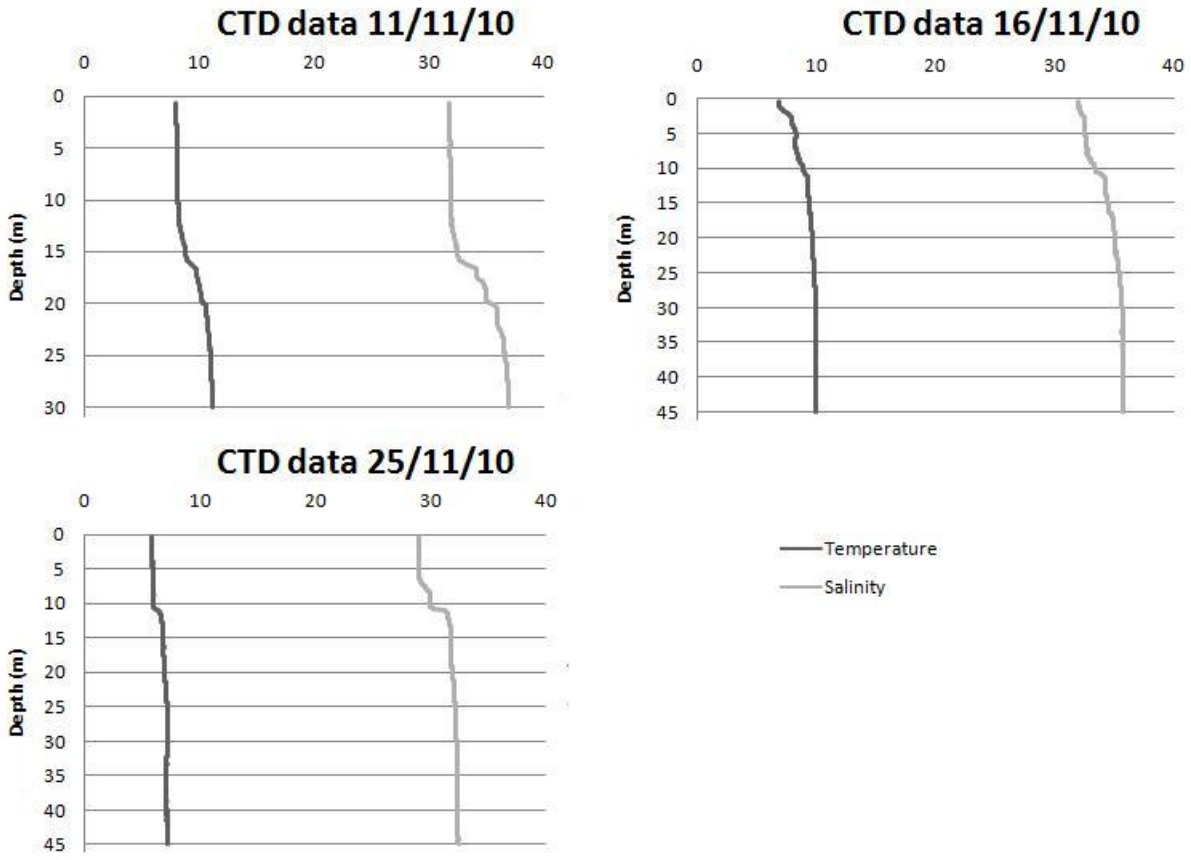


Fig 3.1 CTD profiles from the three surveys. Temperature in °C and salinity in °/oo

3.1.2 11/11/2010 survey [APPENDIX 1]

During the first survey carried out on the 11th of November 2010 there were 2 water masses. A T-test compared the number of individuals caught only in the superficial layer with the number of individuals caught in the whole water column and showed a significant difference (t<0,01), evidence for the presence of individuals in both the layers.

The biovolume density of *M.leidyi* caught in the upper layer and the one caught in the deeper layer were not statistically different, even if there was a tendency to a greater volume in the deeper water mass (16m-30m). Densities of individuals caught in the superficial layer and in the deeper layer were different (t<0,01).

The size distributions (fig. 3.2a), analyzed with the K-S test, showed a statistical difference (D=0,397, n=13, p<0,05).

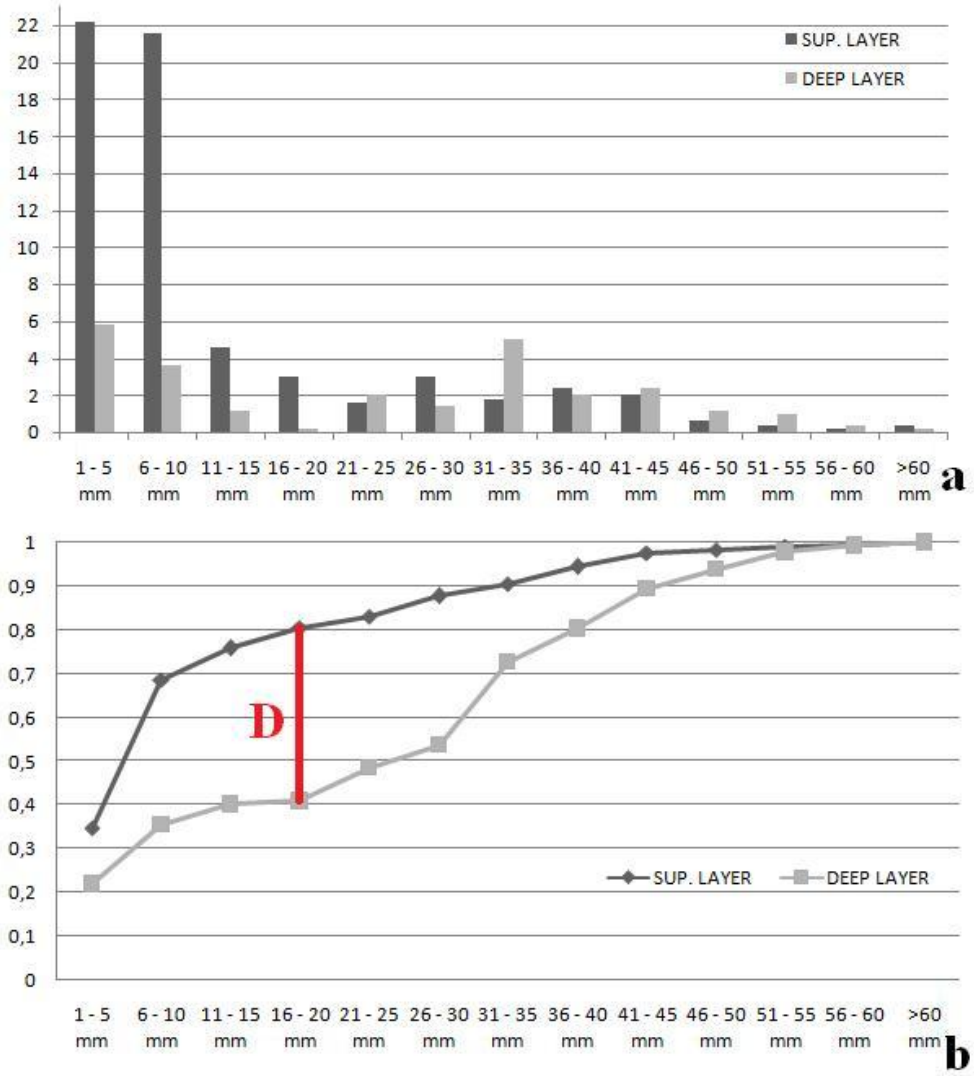


Fig. 3.2 (a) Size distribution of individuals in the two layers of the water column on the 11/11/2010; (b) cumulative frequencies distributions and maximum difference (D) used for the Kolmogorov-Smirnov test.

Analyzing the differences between the layers for every size class, it was possible to detect a statistically higher density in the superficial layer for the classes 0-5mm (t<0,05), 6-10mm (t<0,001) and 16-20mm (t<0,01). The density of the individuals 11-15mm was also higher in the upper layer, but the difference was not statistically significant and it was just a tendency. The class 31-35mm had a significant difference in its density (t<0,05), with a higher value in the deeper layer. The classes from 41mm to 60mm showed a tendency, not statistically proved, for higher densities below 16m of depth.

During this survey an average of 3.2 individuals of Cyanea capillata had been caught in the superficial layer (density=0,26 ind/L) and 2,2 individuals from the whole column (0,17 ind/l).

3.1.3 16/11/2010 survey [APPENDIX 2]

Three strata were found in the water column on the 16th of November during the second survey. The presence of *Mnemiopsis leidyi* below the most superficial layer (t-tests comparing the superficial layer and the whole column) resulted either from the total number of individuals (t<0,01) and from the biovolume (t<0,001). T-tests between the samples of the superficial layer plus the intermediate layer and the samples from the whole column were also done to detect the presence of *M.leidyi* in the deepest layer (28m-40m). In this case the total number of individuals caught is not statistically different (t=0,23), but the biovolume shows a significant difference (t<0,05), showing the presence of the combjellies also in this deepest layer.

The density of ctenophores was statistically different between the deepest and the most superficial layer as showed by the density of individuals (t<0,05) and by the biovolume density (t<0,05). The intermediate layer was different from the most superficial layer only in the density of individuals (t<0,05) and was not statistically different from the deepest layer.

The size distribution was different between superficial and deepest layer (D=1,580, n=13, p<0,01), while the intermediate layer had a distribution statistically different only from the deeper layer (D=1,359, n=13, p<0,05) (fig.3a).The density of the size classes between the surface and the deepest layer was statistically different for the class 16-20mm (t<0,05) with a higher density in the surface layer and for the classes 36-40mm (t<0,05), 46-50mm (t<0,001), 56-60mm (t<0,05) and >60mm (t<0,001) with higher densities below 28m of depth. The two smallest classes (0-10mm) were not present in the deepest layer (average negative density) while the two biggest were not in the superficial one.

Comparing the intermediate layer with the superficial one, the only significant result was for the class >60mm (t<0,05), but there was a clear tendency for the smaller classes to have a higher density in the surface and for the bigger classes to be more present in the intermediate layer. Compared with the deeper layer, in the intermediate layer the class 0-5mm had statistically higher density (t<0,05), while classes 21-25mm (t<0,05), 36-40mm (t<0,05), 46-50mm (t<0,05) and >60mm (t<0,05) were less dense. The four smallest classes (0-20mm) tended to be more concentrated in the intermediate layer while the seven biggest classes (30->60mm) tended to higher concentrations in the deeper layer.

Just two individuals of *Cyanea capillata* were present in the samples, one from a catch from the intermediate plus superficial layer (45mm wide, 3ml of biovolume) and another in the sample from the whole column (64mm wide, 11ml of biovolume). One *Beroe cucumis* 48mm long were caught in one sample from a vertical haul from the bottom of the intermediate layer.

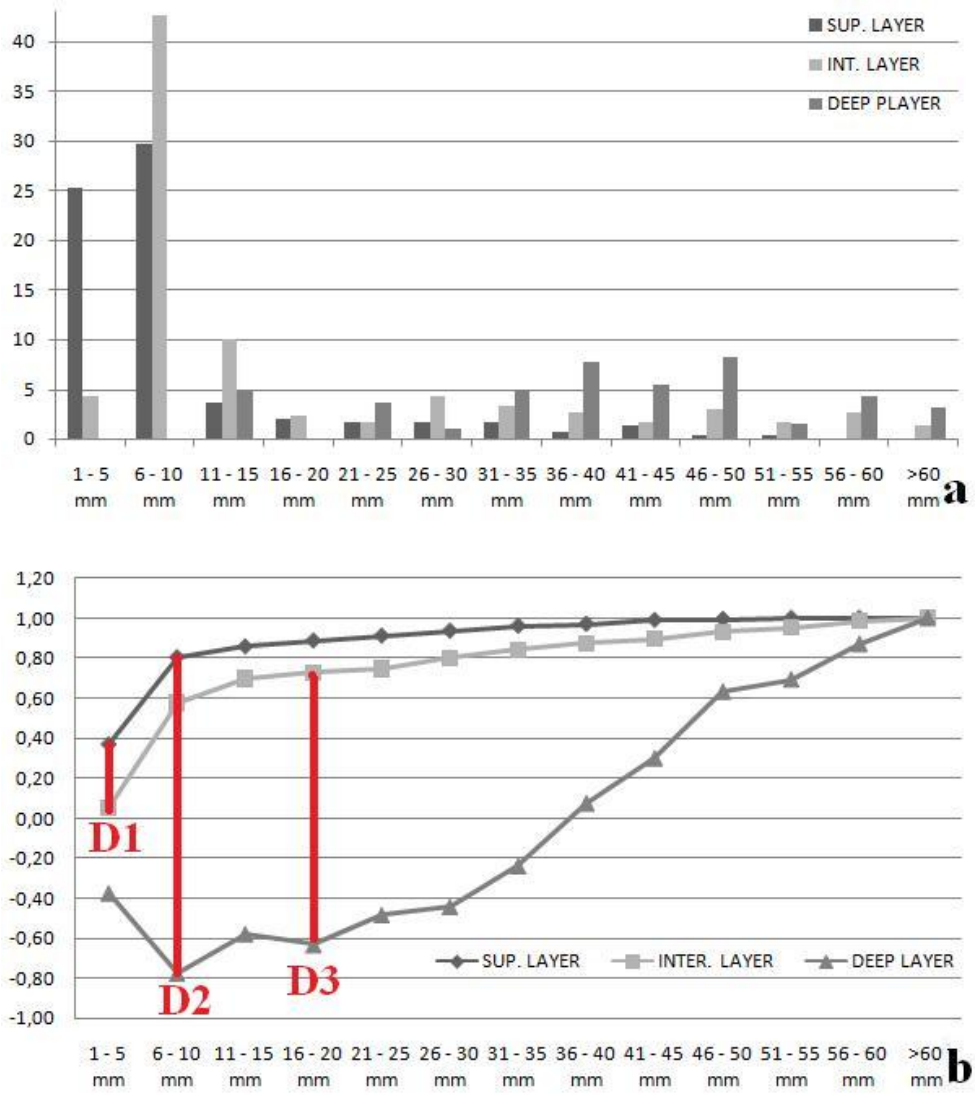


Fig. 3.3 (a) Size distribution of individuals in the three layers of the water column on the 16/11/2010; (b) cumulative frequencies distributions and maximum differences (D) used for the Kolmogorov-Smirnov test. D1: max difference between superficial and intermediate layer; D2: max difference between superficial and deep layer; D3: max difference between intermediate and deep layer;

3.1.4 25/11/2010 survey [APPENDIX 3]

The third survey, on the 25th of November, was carried on a 2 layers system. The presence of *M.leidyi* in both the water masses was showed by the significant difference between the number of individuals caught in the superficial layer and the ones caught in the whole column ($t<0,001$). The same was valid also for the biovolume ($t<0,001$).

The size distribution in size classes (fig. 3.4a) was not different between layers.

The densities of individuals and biovolume of combjellies were not statistically different in the superficial and deep layers. Analysis on the size classes showed only one significant output, the class 36-40mm ($t<0,05$) with higher density in the deeper layer. There were no clear tendencies.

An average of 1 *Cyanea* per sample was detected in the samples from the whole column with a size range 3-8cm. In the surface only two individuals were caught, both of them 7cm wide.

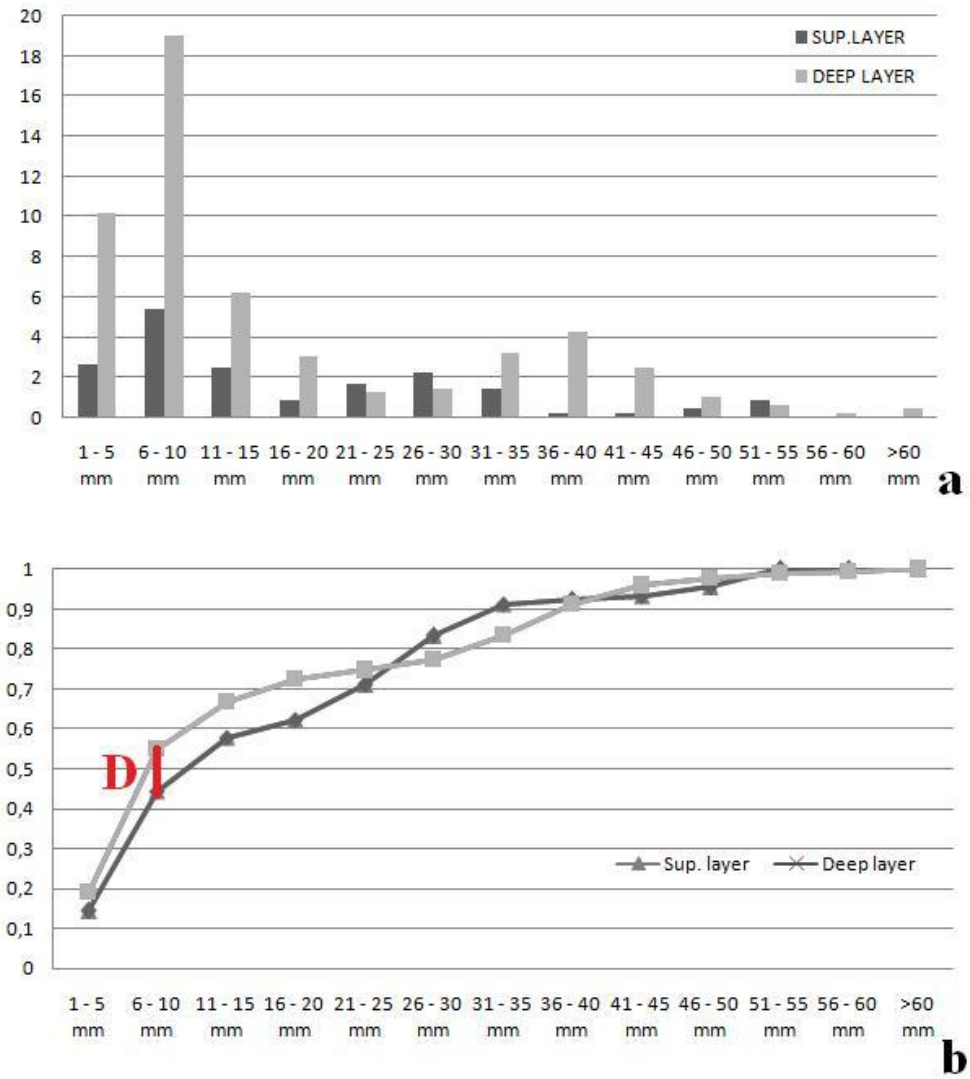


Fig.3.4 (a) Size distribution of individuals in the two layers of the water column on the 25/11/2010; (b) cumulative frequencies distributions and maximum difference (D) used for the Kolmogorov-Smirnov test.

3.1.5 Zooplankton composition [APPENDIX 4]

Ten classes of zooplankton (among different taxa or different developmental stages) were chosen to analyze the plankton.

The cladocerans were always present in the superficial layer, but not in the deeper ones, where only one individual was caught during the third survey.

The copepods belonging to the species of *Microsetella norvegica* had, instead, lower densities in the superficial layer than in the deeper water masses. This was statistically confirmed in the first ($t<0,05$) and second survey ($t<0,01$), where these copepods were more concentrated respectively in the deepest and intermediate layer. During the third survey this difference was just a tendency not statistically significant. Rotifera showed the same pattern, with higher densities in the deepest layer in the first survey ($t<0,001$) and in the intermediate layer in the second ($t<0,01$) and with no difference at the time of the third survey.

The barnacle larvae showed a bloom from the middle of November involving only the superficial layer, where there was higher density than in the deeper masses in the second ($t<0,001$) and in the third surveys ($t<0,01$). The density of individuals was increasing from 0,22 individuals/liter on the 11/11/10 to 0,76 ind/l on the 16/11/10 to 2,86 ind/l on the 25/11/10.

The copepod nauplius larvae, had highest densities in the superficial layer in the first (t<0,001) and second surveys (t<0,01), but they were more concentrated in the deeper layer on the third one (t<0,05), where there was the highest density recorded with 5,47 individuals/liter.
The copepodites, the developmental stages after the nauplii and before the adults, were more concentrated in the deeper layer in the first survey (t<0,01), in the superficial layer in the second survey (t<0,05) and again in the deeper water in the third survey (t<0,001), where the highest density was found (1,47 ind/l) like for the nauplii.
The adult copepods showed no statistical differences between water masses. Individuals of genus *Acartia sp.*tended to be more concentrated in the deeper layers in the first two surveys and in the superficial layer in the third one but no statistical evidence of this difference was found. The adults of genus *Oithona* presented instead a statistical difference during the third survey, with a bloom involving only the deeper layer (t<0,01). In the previous two surveys they tended to be denser in the superficial layer of the first survey and in the deeper layers of the second one (tendencies not statistical proved).
During the sampling period a phytoplankton bloom occurred. There were algae belonging to different genera like *Rhizosolenia, Ceratium, Protoperidinium, Coscinodiscus* and *Dinobryon* in really high densities. The estimation of the abundance of these species went beyond the aim of this study and was not carried on. This phytoplankton mass could have affected the zooplankton analysis on the field clogging the net and the pump and in the lab forcing to dilute the samples more times and hiding the smallest zooplankton during the counting.

3.2 Belgium – 2011 – genetic study

3.2.1 ITS

A total of 208 samples were sent to ILVO in Oostende. Molecular identification (fig. 3.5; APPENDIX VI ITS) targeted 105 of them as *Mnemiopsis leidyi*, 46 as *Beroe cucumis*, 11 as *Pleurobrachia pileus*, 8 as *Lovenella assimilis*, 4 as *Beroe ovata*, 2 as *Bolinopsis infundibulum* and 2 *Velamen parallelum*. For 19 samples the DNA extraction failed, while 8 times the sequencing process failed or, for 2 samples, sequence quality was too low to rely on the results of the nucleotide BLAST query. For every sample the parameters query coverage, e-value and max identity were reported after.
A first visual identification based on morphology was done soon after the catching for 174 samples. In 18 cases specimen first identified as M. leidyi turned out to belong to different species, while in 4 cases M. leidyi had been initially misidentified.

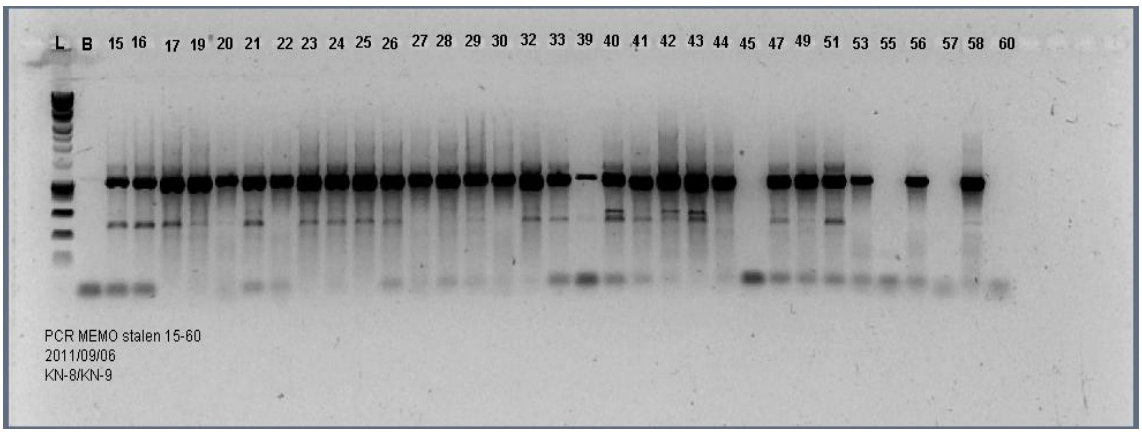


Fig. 3.5 Agarose gel electrophoresis showing PCR products after amplification with primers KMBN-8 and KMBN-9 for ITS region.

The analysis on the two Italian samples identified the two jellyfish as *Mnemiopsis leidyi* (First sample: query coverage 100%, e-value 0, max identity 100%; second sample: query coverage 99%, e-value 0, max identity 99%). This is the first molecular identification reported about samples caught in Italian waters (fig. 3.6) (Binetti et al., in press).

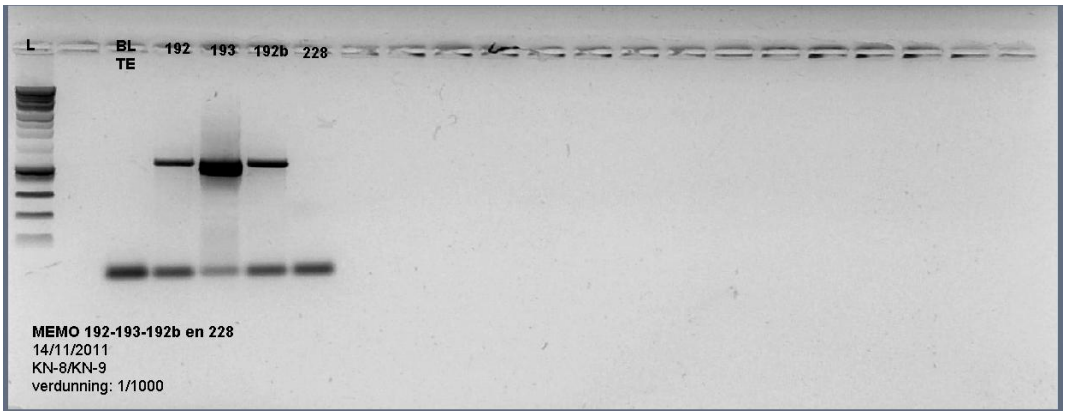


Fig. 3.6 Agarose gel electrophoresis showing PCR products after amplification with primers KMBN-8 and KMBN-9 for ITS region on Italian samples.

Among the 105 *M. leidyi* identified, 4 individuals showed an higher grade of variation than the other 101. In these 4 specimens, 41 positions had a different base than the other 101 samples. All these positions varied in all the four individuals and in all of them the substitution was with the same new base. Other 24 positions showed variation, but they were located at the beginning and at the end of the string and the sequence quality was too low showing these 24 variations just in one or two individuals out of the anomalous four.

	ITS1			ITS2			
ALLELE	64	193	212	416	507	574	583
A	C	A	A	A	C	A	C
B	C	A	T	G	C	A	C
C	C	G	A	G	C	A	C
D	C	A	A	G	C	A	C
E	T	A	A	A	C	A	C
F	C	A	T	A	C	A	C
G	C	A	A	G	T	A	C
H	C	A	T	G	C	T	C
I	C	A	T	G	C	A	A
J	C	G	A	A	C	A	C
K	C	A	T	A	C	A	A
L	T	A	T	G	C	A	C
M	T	A	A	G	C	A	C
NEW ALLELE	C	G	A	G	C	G	C
ANOMALOUS	C	A	G	G	C	G	C

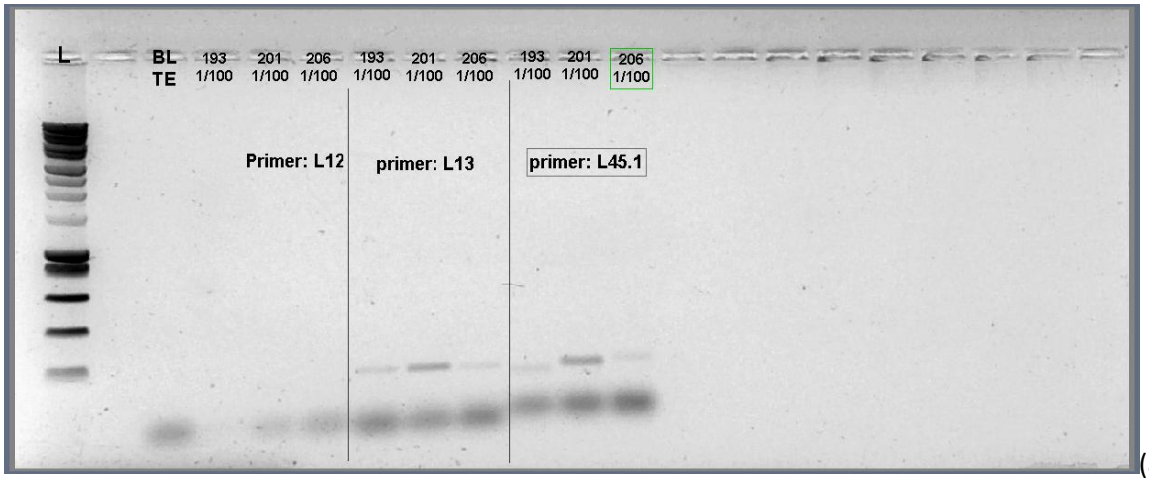
Tab. 3.1 Alleles of the ITS region for *M. leidyi* after Ghabooli et al. (2010) with the 2 new ones discovered in this study. For each allele are presented the bases in the diagnostic positions.

according to Ghabooli et al. (2010), 13 alleles in the ITS region are present in *Mnemiopsis leidyi* populations (allele from A to M). These 13 alleles are discerned by 7 positions in the sequence. 37 individuals resulted homozygous for allele C (Genbank accession number GU062752.1), 1 for allele B (GU062751.1) and 1 for allele H(GU062756.1). 22 individuals were heterozygous, as shown by the ambiguities in those positions. Following the position numbers of Ghaboli et al. (2010), 13 individuals had ambiguities R in position 193 and W in position 212, 11 had R in position 193 and in position 416 and 4 had W in position 212 and R in position 416, 1 had just R in position 193 and 4 had just R in position 416. Moreover, 33 strings lack some of these diagnostic bases due to low quality of the sequencing. For 21 sequences it is unknown the base in position 64, for 18 samples the base in position 193, for 12 in position 212 and for 12 the bases in positions 574 and 583.

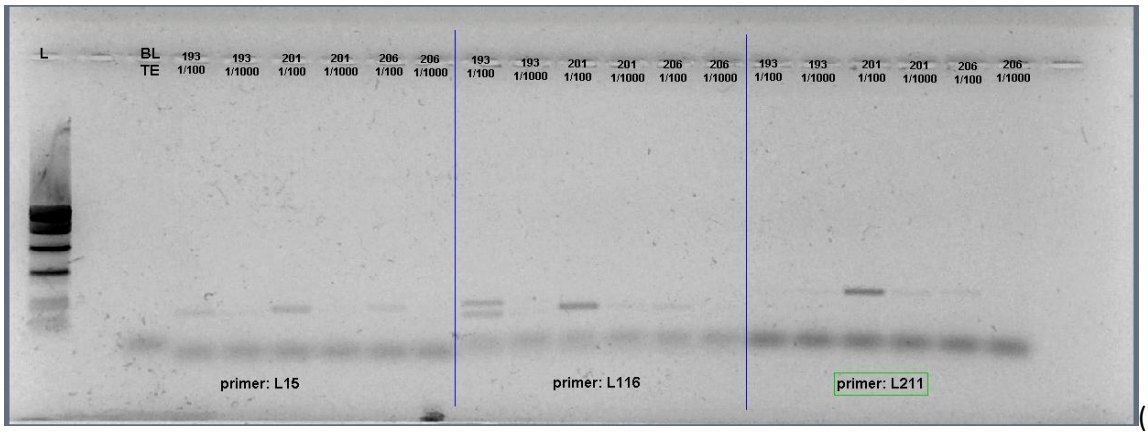
Two samples were found homozygous for a new allele not reported in the previous study. It resembled the allele C of ITS, but it had a G in position 574 instead of A. G was a new base for this position, since only A and T had been described in Ghabooli et al. (2010). Also the four anomalous sequences had a new allele with a G in position 574, but also with a G in position 212 where only A and T had been previously reported (Tab. 3.1)

3.2.2 Microsatellites

The optimization of the protocol was focused on testing that the primers worked effectively. All microsatellites but C1583 were tested singly. In the Pool1, L12 did not show clear bands after the amplification (fig. 3.7a). Primers of Pool2 gave bands for all the three microsatellites, but less visible in L15 (fig 3.7b).



(a)



(b)

Fig. 3.7 Agarose gel electrophoresis showing PCR products after amplification with microsatellites primers of Pool1 (a) and Pool2 (b).

After having use different annealing temperatures in the PCR protocol of the two pools after Reusch et al.(2010), multiplex PCR was successfully performed for both the pools with the same annealing temperature of 60 °C. Pool2 worked better with clearer bands in the gel. Different bands in the same individual means different length of microsatellites amplified (fig. 3.8).

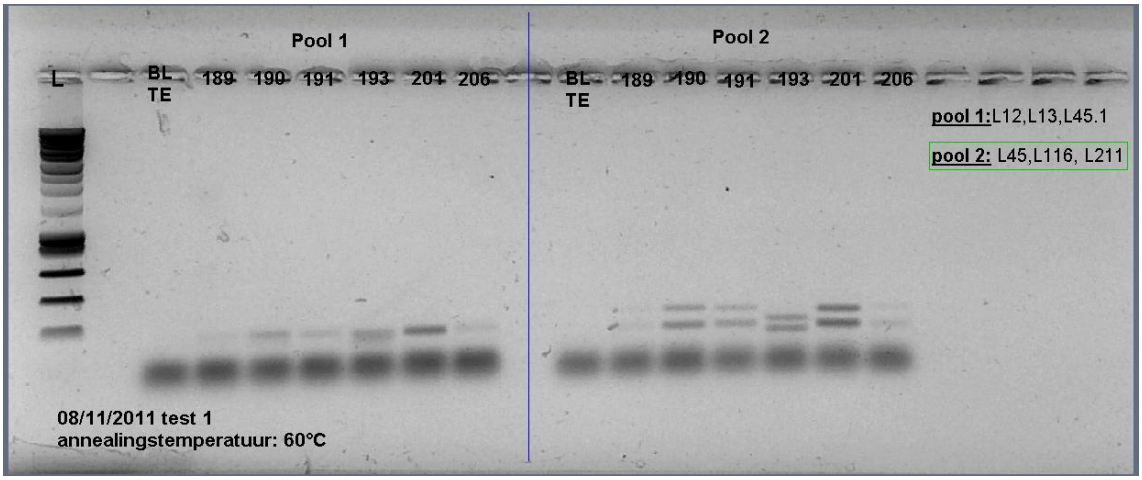


Fig 3.8 Agarose gel electrophoresis showing results of the Multiplex PCR. Microsatellites are divided in 2 Pools (legend).

The last part of the optimization was thee increasing in the DNA content in the vials (fig. 3.9). The highest concentration of DNA was the best in both the pools and then it was used to amplified all the samples for the successive genotyping.

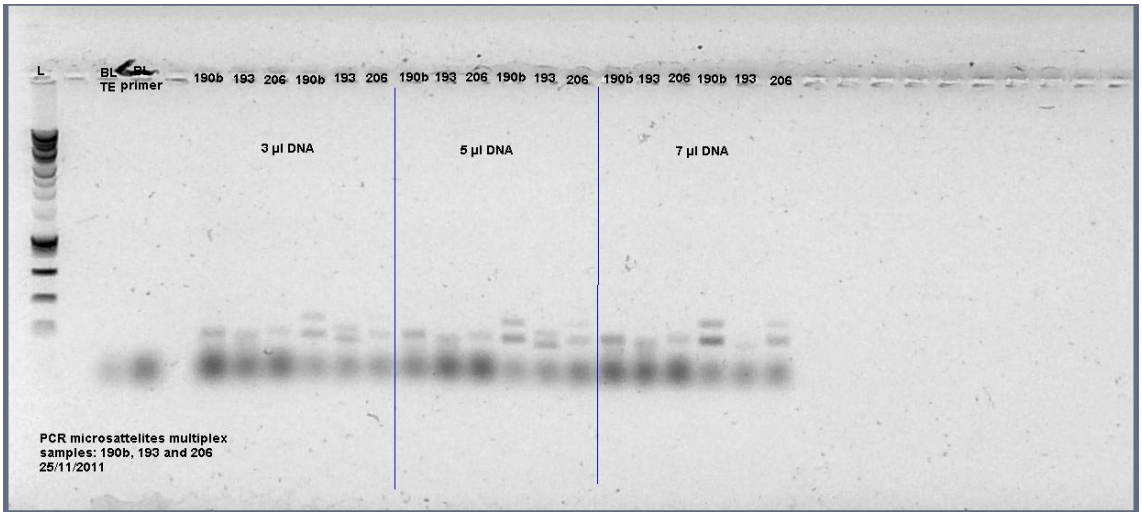


Fig 3.9 Agarose gel electrophoresis showing Multiplex PCR products for 3 different volumes of template DNA (3-5-7µl). For each concentration, former 3 samples are with Pool1 primers and last 3 samples with Pool2 primers.

After the scoring of the peaks, 8 alleles were found for the microsatellites L12, 4 alleles for L13, 11 for L15, 27 alleles for L45.1, 16 for L116, 8 for L211 and 4 alleles for microsatellite C1583. The most diverse locus was L45.1 in all the population but in number 8 where it was L211. Among populations, the one showing the highest average gene diversity was group number 8 (Tab 3.2).

Gene diversity per locus and population :										
C 1583	NA	0,2	0,667	0	NA	0,177	0,497	0,833	NA	0,657
L 116	NA	0,625	0,741	0,833	NA	0,645	0,683	0,833	NA	0,865
L 15	NA	0,825	0,688	0,667	NA	0,778	0,857	0,833	NA	0,8
L 211	NA	0,65	0,875	1	NA	0,718	0,829	1	NA	0,875
L 12	NA	NA	NA	NA	NA	0,833	0,763	NA	NA	0,794
L 13	NA	0	0,143	0,333	NA	0,382	0,498	0,667	NA	0,516
L45.1	NA	0,925	0,917	1	NA	0,928	0,923	0,833	NA	0,931

Tab 3.2 Gene diversity over loci obtained by FSAT for population with more than 1 individual.

The average amount of alleles in each population was correlated with the number of individuals in that population (fig. 3.10). The bigger the sample group, the higher the number of alleles detected in it. The correlation was:

Average number of alleles= 0,307*(numerosity of group)+1,513 (R^2=0,927)

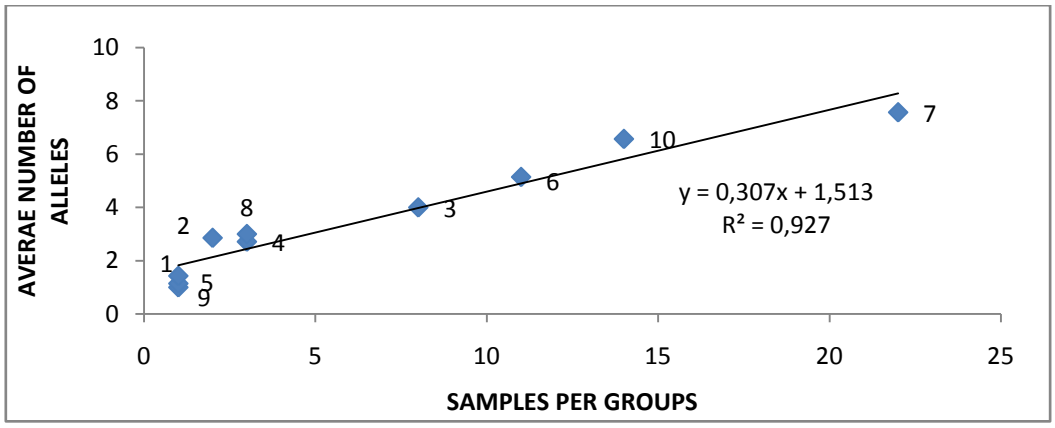


Fig 3.10 Graph showing the correlation between dimension of groups and the average number of alleles over loci.

An AFC plot showed a difference between population 9 and the other groups (fig. 3.11).

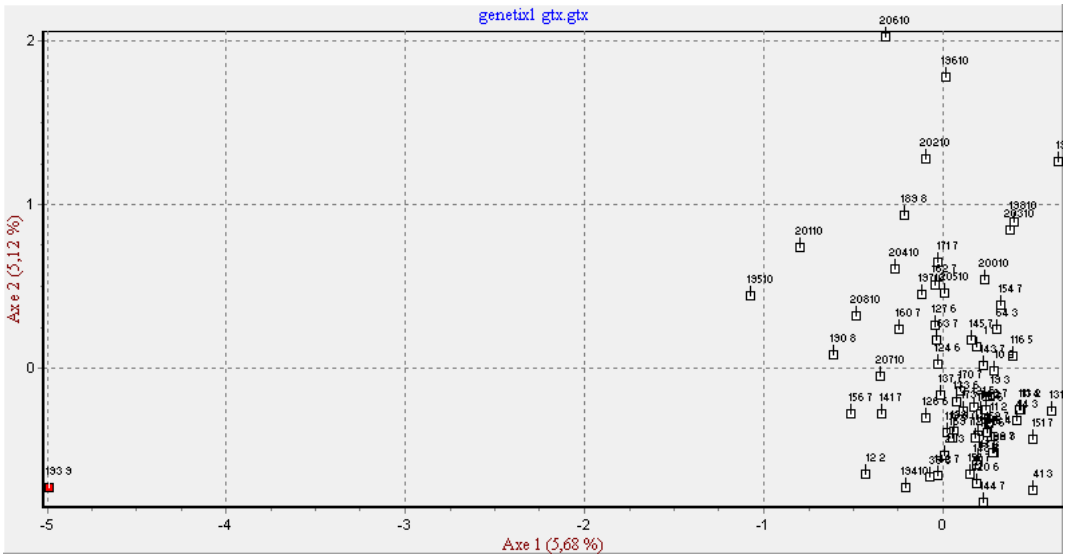


Fig. 3.11 AFC plot from GENETIX showing spatially differences among populations based on microsatellites alleles frequencies. Red dot is population 9, Italian sample. The other 10 population from North Sea are colorless dots.

This pattern was confirmed calculating the pairwise distance (Cavalli-Sforza and Edwards, 1967)among populations with group 9 having the highest values (Tab 3.3).

	1	2	3	4	5	6	7	8	9	10
1 (1)	0.000	0.192	0.225	0.180	0.186	0.113	0.155	0.271	0.355	0.180
2 (5)	0.192	0.000	0.156	0.125	0.155	0.136	0.138	0.248	0.328	0.191
3 (8)	0.225	0.156	0.000	0.128	0.213	0.124	0.126	0.233	0.335	0.198
4 (3)	0.180	0.125	0.128	0.000	0.190	0.117	0.114	0.246	0.362	0.190
5 (1)	0.186	0.155	0.213	0.190	0.000	0.196	0.193	0.278	0.394	0.225
6 (11)	0.113	0.136	0.124	0.117	0.196	0.000	0.057	0.177	0.318	0.120
7 (22)	0.155	0.138	0.126	0.114	0.193	0.057	0.000	0.159	0.316	0.101
8 (3)	0.271	0.248	0.233	0.246	0.278	0.177	0.159	0.000	0.325	0.132
9 (1)	0.355	0.328	0.335	0.362	0.394	0.318	0.316	0.325	0.000	0.306
10 (14)	0.180	0.191	0.198	0.190	0.225	0.120	0.101	0.132	0.306	0.000

Tab 3.3 Pairwise distances among populations according to Cavalli-Sforza and Edwards (1967) from GENETIX.

F-statistics parameters according to Weir & Cockerham (1984) among loci were calculated in FSTAT with Jackknife over populations (Tab 3.4). Parameters could not be calculated for L12 because jackknife values simulated monomorphism.

LOCUS	Fit (F)	Fst (Θ)	Fis (f)
C1583	0,400 (0,084)	0,080 (0,086)	0,356 (0,123)
L116	0,290 (0,091)	0,097 (0,074)	0,215 (0,095)
L15	0,288 (0,074)	0,046 (0,043)	0,252 (0,062)
L211	0,399 (0,093)	-0,004 (0,033)	0,402 (0,100)
L12	Not calculated	Not calculated	Not calculated
L13	0,073 (0,156)	0,097 (0,076)	- 0,034 (0,103)
L45.1	0,127 (0,071)	0,027 (0,144)	0,103 (0,075)
OVER ALL LOCI	0,357 (0,085)	0,051 (0,016)	0,323 (0,094)

Tab 3.4 Weir & Cockerham (1984) estimation of Fit (F), Fst (Θ) and Fis (f) over loci by jackknifing over populations. From FSTAT

Hardy-Weinberg equilibrium for population was tested with GENEPOP considering the p-values of the inbreeding coefficient. Populations 3, 6, 7 and 10 were not in HW equilibrium (Tab 3.5).

Population	2	3	4	6	7	8	10
p-value	0,134	0,002 **	0,116	0,002 **	High. Sign. ***	0,145	High. Sign. ***

Tab 3.5 P-values for Hardy-Weinberg probability test with Fisher's method. Values obtained from GENEPOP

Considering only populations 2,3,4,6,7,8 and 10, consisting of more than 1 individual, a matrix of pairwise Fst between groups was obtained from FSTAT and used in STATISTICA to draw a MDS plot (fig. 3.12). This headlines a difference among populations 8 and 10 on one side and all the rest to the other side. The stress value of the MDS plot was 0,004, lower than the threshold of 0,024 that is the acceptable stress level after Sturrock and Rocha (2000) for 2D MDS plots.

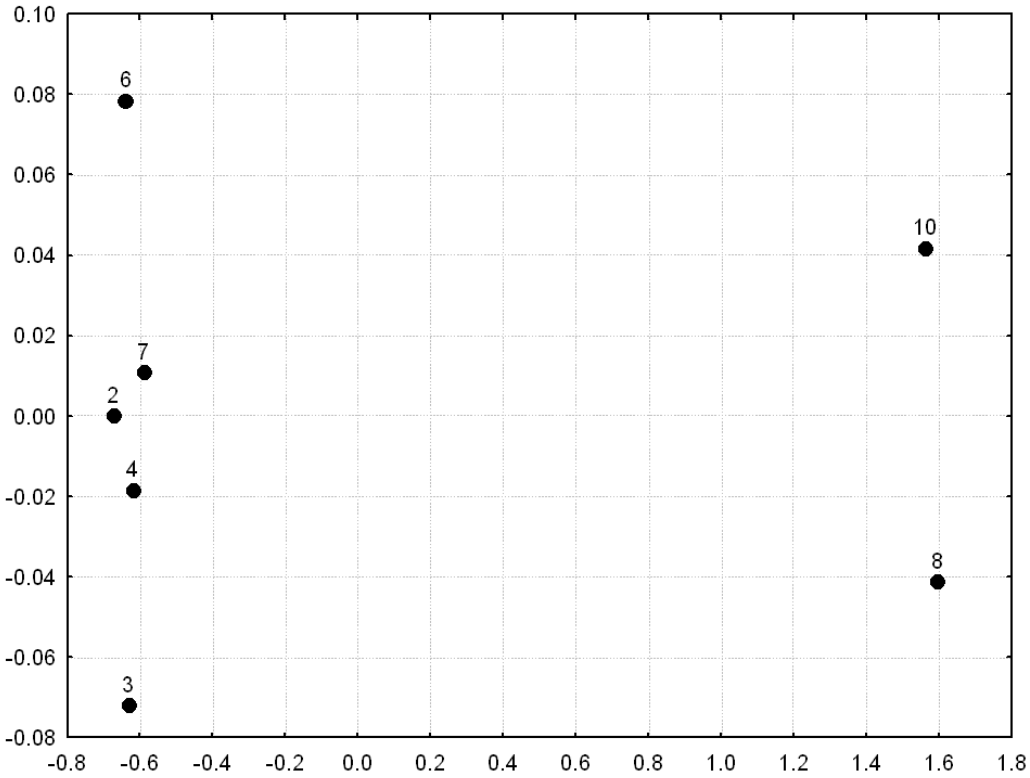


Fig. 3.12 MDS plot from STATISTICA showing spatially the distances among populations with more than 1 individual based on microsatellites alleles frequencies.

The same pattern was found in the bar plot of STRUCTURE. The simulations run testing K subclusters from 1 to 7 were analyzed with STRUCTURE HARVESTER. The DeltaK graph according to Evanno et al. (2005) showed K=2 as the most likely number of subclusters in the overall population (fig. 3.13).
Two sub-clusters were identified, one with populations 2,3,4,6 and 7 (Microsat. Pop. in APPENDIX V) and another with populations 8- and 10 (fig. 3.14).

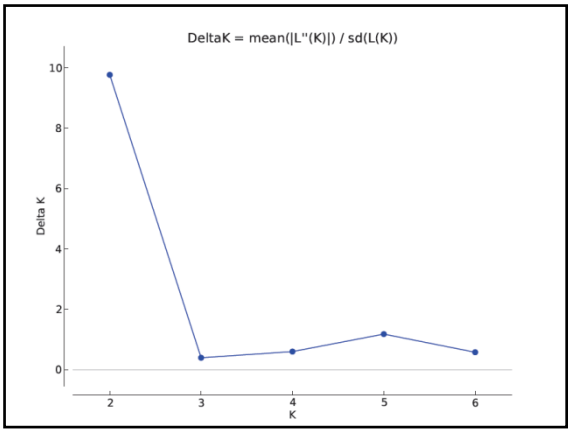


Fig. 3.13 DeltaK graph after Evanno et al. (2005) from STRUCTURE HARVESTER showing the most reliable number of subclusters (K) among samples.

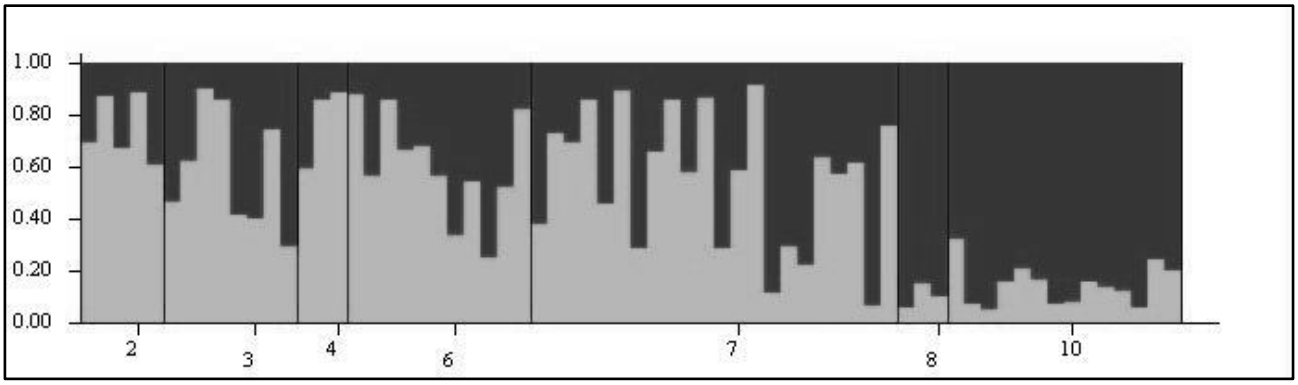


Fig. 3.14 Bar plot from STRUCTURE showing for each individual the probability to be assigned to one of the 2 subcluster considered (K=2). Two subclusters are population 2-7 and population 8-10.

3.2.3 Cytochrome B (cytb)

Primers KMBMT-80 and KMBMT-116 after Bayha (2005) amplified a 361 bp region in the mitochondrial gene Cyt b. The PCR successfully worked on DNA diluted 1/100 and 1/1000 over the original concentration (fig. 3.15; APPENDIX VI CYTB)

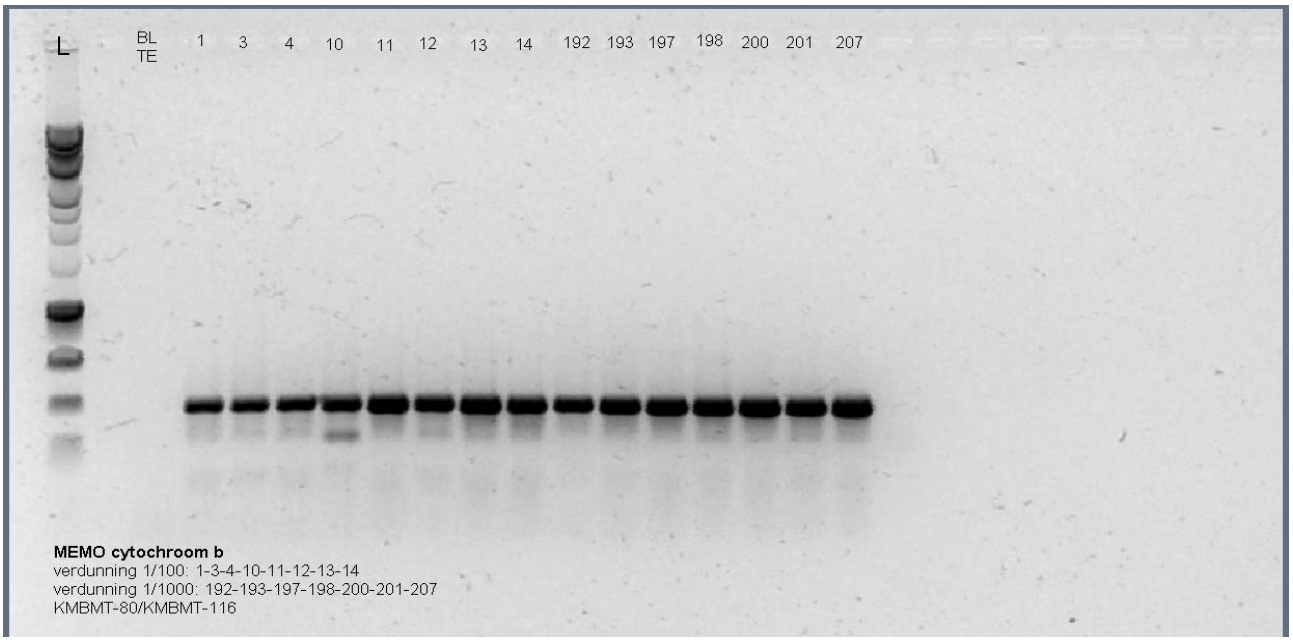


Fig 3.15 Agarose gel electrophoresis showing PCR products after amplification with primers KMBMT-80 and KMBMT-116 for cytb region.

76 sequences were obtained and aligned with BioEdit, showing several positions with varying basis (SNPs). On the purpose of identifying variable positions of the sequence were considered only SNPs with a Minor Allele Frequency (MAF) of 5% and whose position had been successfully amplify on at least 70% of the sequences (coverage 70%).Complying with position numbers of the mtDNA after Pett and Ryan, 2011 (GenBank acc.num. JF760210), two true SNPs were identified in position 9307 (58%T, 29%C, 13% ambiguity Y, coverage 100%) and 9539 (78% A, 18% T, 4% ambiguity W, coverage 72%). Despite the parameters chosen to recognize a Single Nucleotide Polymorphism, other positions in the sequence have high variability. Position 9463 is a candidate SNP, since the MAF is 4%, lower than 5% picked as threshold (96% T, 4% C, coverage 99%). Positions 9190 (59% C, 41% Y, coverage 100%), 9256 (59% C, 41% Y, coverage

100%) and 9224 (75% C, 25% Y, coverage 100%) show the same ambiguity in a consistent number of sequences. Also position 9204 present a candidate SNP (96% A, 3%G, 1% ambiguity W, coverage 100%) with base G present only in the Italian samples.

After the publication of the mtDNA a new primer has been used to amplify a broader region. Analyses on OligoAnalyzer gave a melting temperature of the hairpin conformations lower than the annealing temperature used in the protocol. Every possible self-dimer was associated to a value of variation of free energy Delta G that was compared with the Maximum Delta G. The higher Delta G value was 7 times smaller than the Maximum, showing little influence of self-dimers in the PCR reactions. The same calculation for hetero-dimers with the primer KMBMT-116 gave the highest Delta G more than 5 times smaller than Maximum. Three sequences were obtained using the new primer (fig. 3.16). For two samples out of three the new sequence was actually longer than the one obtained with the primers from Bayha (2005).

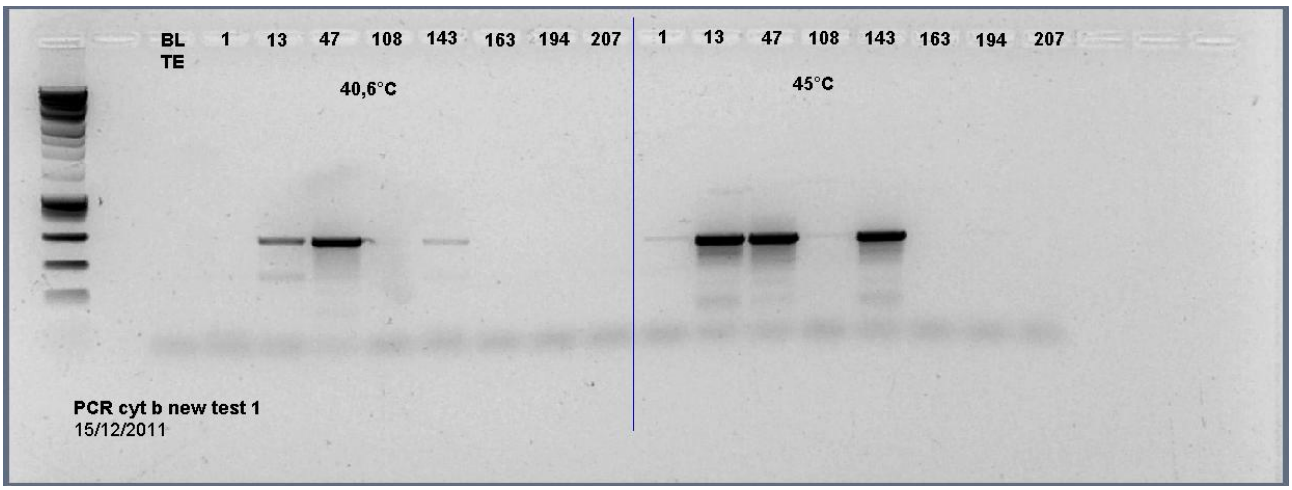


Fig. 3.16 Agarose gel electrophoresis showing PCR amplification with new reverse primer and KMBMT-116 after Bayha (2004) with two different annealing temperatures.

3.2.4 Cythochrome C oxidase subunit 1 (cox1)

Two new primers were designed to amplify a 787 bp region in the region cox1 of the mtDNA. Both of them were tested on OligoAnalyzer. The melting temperature of the hairpin conformations for both of them were lower than the annealing temperature used in any protocol. Self-dimers were also considered comparing the variation of free energy (Delta G) of every possible self-coiling conformation with a Maximum Delta G. Both the primers had the highest Delta G of their self-dimers smaller than 1/3 of the Maximum Delta G. The same criterion was used to calculate the hetero-dimers between the two primers and the Maximum Delta G was more than 10 times greater than the highest Delta G associated to each possible hetero-dimer.

Once tested virtually, two primers were tested by real trying to optimize the PCR protocol and the concentration of the PCR solution components. The PCR worked with an annealing temperature range from 35 °C to 51 °C, but failed at a temperature of 55 °C. Only one sample out of two worked (fig. 3.17).

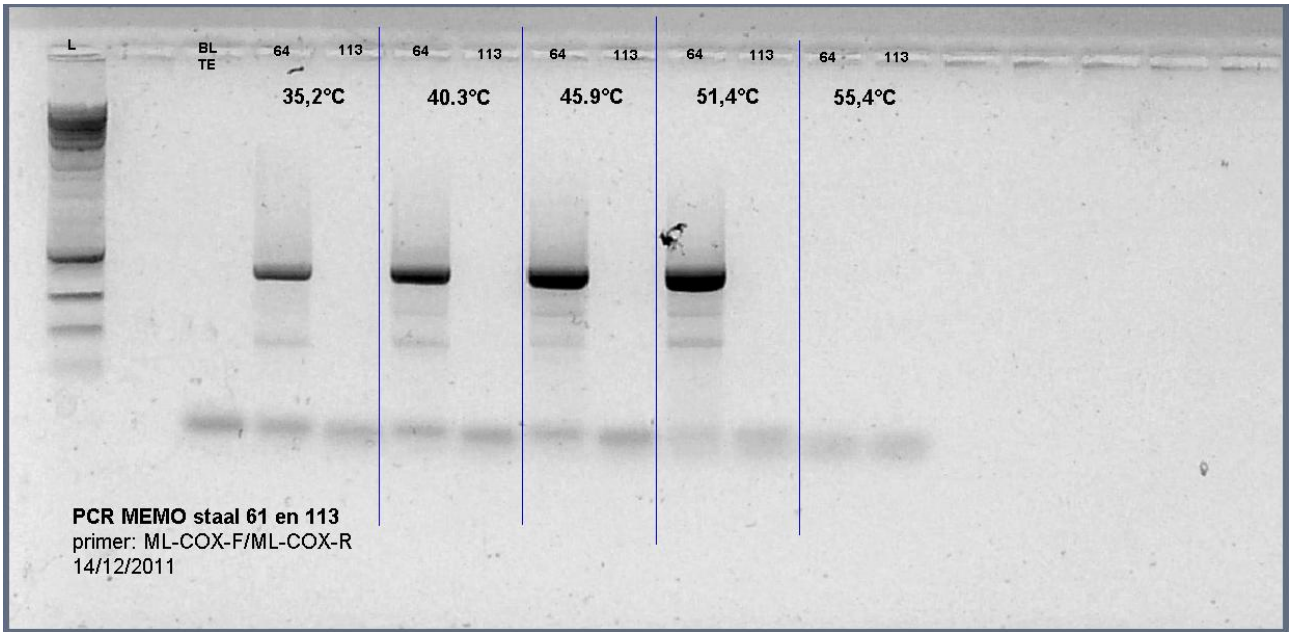


Fig. 3.17 Agarose gel electrophoresis showing PCR products after amplification with primers for cox1 region with 5 different annealing temperatures.

This sample was tested again with other two ones changing temperature and Mg^{++} concentration in the PCR solution (fig. 3.18). Only the same sample used in the previous test worked. Adding $MgCl_2$ a second band compared in the gel. A temperature of 45,9 °C and a Mg^{++} concentration of 1,5mM were chosen as the best parameters and used in the successive analyses.

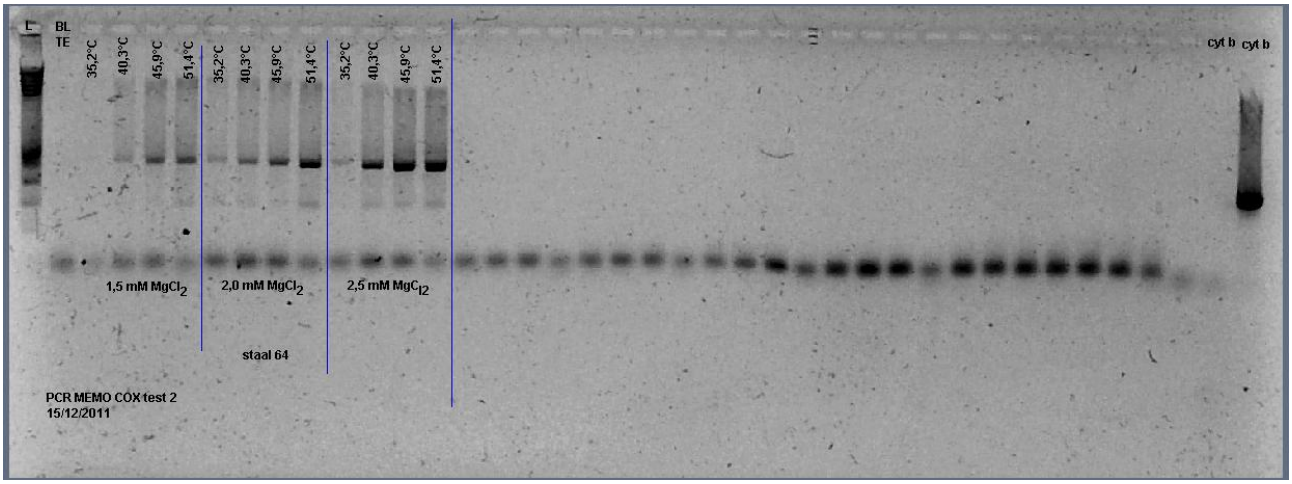


Fig. 3.18 Agarose gel electrophoresis showing PCR products after amplification with primers for cox1 region with 4 different annealing temperatures and 3 $MgCl_2$ concentrations.

Testing more samples with the annealing temperature of 45 °C, the PCR with a Mg^{++} concentration of 2,5mM failed for two samples that were instead amplified with $[Mg^{++}]$=1,5mM (fig. 3.19).

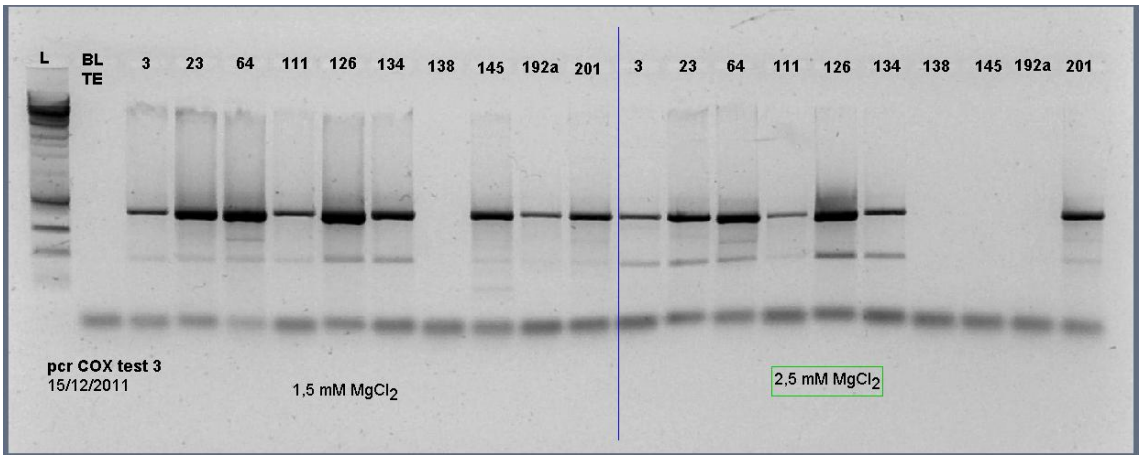


Fig. 3.19 Agarose gel electrophoresis showing PCR products after amplification with primers for cox1 region with two $MgCl_2$ concentrations

Another PCR with annealing temperature of 45 °C and a [Mg^{++}] of 1,5 mM was carried out on 34 new samples and 21 for 21 of them amplification was successful. At the end of tests, 27 sequences were obtained from VIB and were aligned in BioEdit (fig. 3.20).

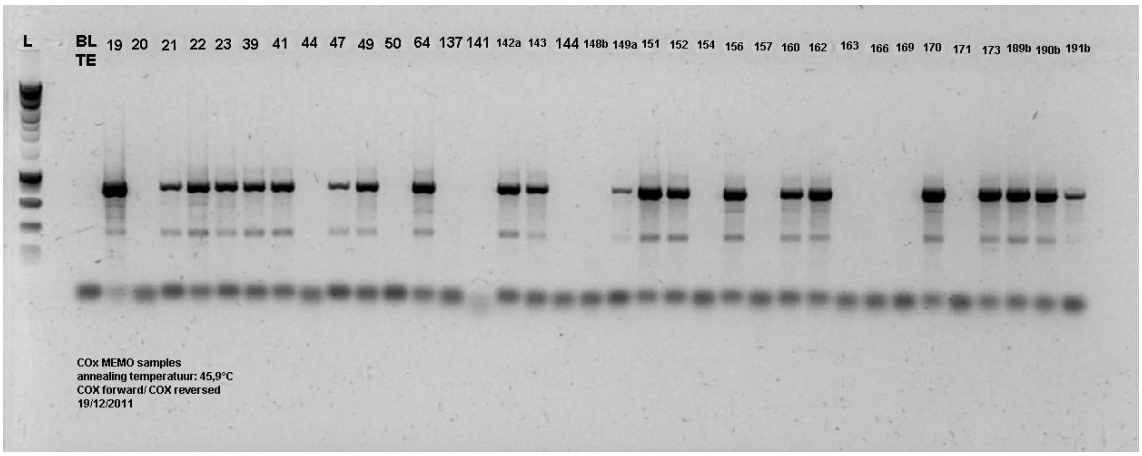


Fig. 3.20 Agarose gel electrophoresis showing PCR products after amplification with new primers designed for cox1 region.

The alignment of the 27 sequences with the cox1 sequence from the mtDNA published on GenBank (JF760210) showed several points of variation. In 4 positions it is possible to suggest the presence of a SNP. Referring to the position numbers of the mtDNA published on GenBank, SNP are present on positions 135 (48% G, 41% A, 11% ambiguity), position 363 (93% T, 7% G), position 435 (52% G, 41% A, 7% ambiguity) and position 502 (89% A, 11% G). Positions with high frequencies of ambiguities (positions 812, 833, 840, 841) were considered candidate SNPs. Deletions are present at positions 93 (59% missing A, not sequenced 7%) and 849 (missing A 33%, not sequenced 11%).

Another test was done to see if these primers were working also on other species than *Mnemiopsis leidyi*. Cox1 was amplified in *Pleurobrachia pileus* and *Lovenella assimilis*, but only on 4 *Beroe cucumis* out of 12 samples with not clear bands and primers did not work on the two *Beroe ovata* samples (fig 3.21).

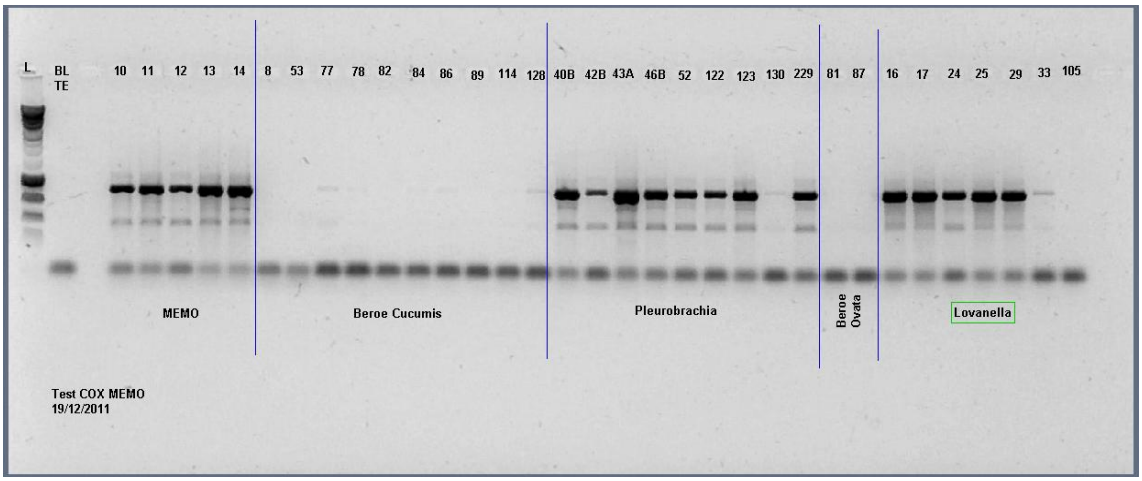


Fig. 3.21 Agarose gel electrophoresis showing PCR products after amplification with primers for cox1 tested on *M. leidyi, Beroe cucumis, Pleurobrachia pileus, B. ovate* and *Lovenella assimilis*.

4 DISCUSSION

Non-endemic species became a major issue in the last decades. Studying just European coastal waters (including navigational inland waterways and adjacent water bodies) and considering taxa as different as unicellular algae, multicellular algae, invertebrates and vertebrates, more than 1000 species have been identified as invasive, half of which successfully settling in the new habitats with self-sustaining populations (Gollasch 2006). *Mnemiopsis leidyi*, listed among the 100 more dangerous invasive species (Lowe et al., 2004), was studied in two different seasons, the last two months of 2010 and the last four months of 2011. The goal of the study was to approach the problem of the ctenophore *Mnemiopsis leidyi* from different points of view in order to have an overall idea about how to conduct studies on invasive species and to become able to gather data to compare with previous studies or to give new info about these organisms in their new invaded habitat. In this perspective, an ecological descriptive approach and a genetic one were followed.

The ecological descriptive survey was carried in Denmark where this lobate ctenophore has been recorded since 2005, with a focus on the Aarhus bay where it is present since 2007 (Tendal et al., 2006). Inner Danish Waters, including the south part of the Skagerrak, the Kattegat and the Danish Straits, constitute a big estuarine system where waters from two different areas, the North Sea and the Baltic Sea converge creating a distinct stratification of the water column. Water masses are obviously different in the CTD profiles and also in their plankton distribution. During the whole sampling time of this experiment the superficial layer was colder and fresher than the deeper one, due to the presence in the surface of water coming from the Baltic Sea, while below a layer with water coming from the North Sea was detectable down to the bottom, a typical situation in autumn season in this area. It was possible to observe between layers either a small pycnocline or, as in CTD profiles of the second survey, a whole interposed layer where surface and deep waters mixed. Also the plankton composition varied between the Baltic and North Sea waters, with copepod *Microsetella norvegica* tending to be more present in the deeper layers of the column (deepest layer in the first survey and intermediate layer in the second one) and cladocerans detected only in the Baltic water (just one individual detected in the deepest layer during the whole experiment).

This is the first study carried on to analyze and to estimate the abundance and distribution of *Mnemiopsis leidyi* in this part of the Inner Danish Water. Considering the differences in the water masses, a difference in the populations of this species was expected at different depths, especially when the column was well stratified as in the first two surveys. During the first sampling, there was a statistically significant difference in the density of individuals caught in the two masses and in the size distributions between populations. More individuals per liter in the superficial layer were not influencing statistically the difference in biovolume. This can be explained by analyzing the differences among the size classes. In fact, only the smallest individuals had higher densities in the surface so the great variation in number due to these small combjellies was related to a small variation in the biovolume between the populations. In the second survey, instead, a difference was present also in the biovolume density between the two layers and, in fact, also bigger size classes had different densities between the superficial and deeper waters as well as small ones. While the variation in the density of individuals is due to lot of small individuals in the surface, the difference in biovolume between layers is due to few big combjellies in the deep water mass. The intermediate level of this second survey was a mix of the two layers as showed by the presence of the big individuals (>60mm), absent in the superficial layer, and by the presence of the smallest ones (<10mm), absent deeper. Moreover, an analysis of the size distributions with the K-S test showed that this layer was

more influenced by the superficial layer. Populations of the two most superficial layers, in fact, seem to have the same class distribution, while they are different between the intermediate and the deepest layer.

The third survey presented a more mixed column, as shown by temperature and salinity that varied more smoothly between layers than during the other surveys. In this water column there seemed to be only one population of *M. leidyi* with no depth-dependent differences. The homogeneity in size distributions showed by K-S test was another evidence of this unique population in the water column. Also zooplankton composition suggested the mix of water masses, with no difference in density of *Microsetella norvegica* between superficial and deep water and with the only cladoceran recorded in the deepest layer. Cladocerans distribution could be the result of vertical diel distribution (Horppila, 1997), but the presence of only one individual in the deep layer when the column is better mixed can be a clue of the actual presence of mixing forces in the water column that can eventually mix also populations of zooplankters passively segregated in different water masses as ctenophores.

From these data it was thus possible to detect two different populations belonging to the different water masses. In the deep water there was an older population than in the surface, as shown by the presence of more big individuals. The higher abundance of small combjellies in the superficial layer could be instead evidence of a higher reproduction rate for the population in the upper waters.

The plankton analysis showed some correlation with the *Mnemiopsis leidyi* distribution in the water column. In particular there seemed to be a relation between the distribution of the smallest individuals of combjelly and the nauplii, the first stages of the crustacean larvae, supposedly mostly copepod larvae. Water column layers with higher densities of nauplii were the same where there were higher densities of small *M.leidyi*. Since the number of smallest individuals drives the difference about the total number of ctenophores caught in different layers, the abundance of nauplii was related also with the density of individuals of the whole population, which means that comparing different water masses, where more nauplii were caught, there were also more *M.leidyi*. Considering the size of these crustacean larvae, they can be considered a suitable feeding source for the youngest stages of *M.leidyi*. Moreover, this class of zooplankton was by far the one with highest density among the zooplankton classes here considered and so it may be seen as a good indicator of the food availability. Food abundance could be an important factor to explain the differences in density of *M.leidyi* between the water masses, considering also the quick reaction that the populations of this ctenophore have in relation to the food abundance blooms (Purcell et al, 2001). Other important influences could be the burst of barnacles in the superficial layer in the second and third surveys or bloom of rotifers, especially in the deeper layers where they had higher densities. Copepodites in the second and third survey were more concentrated in the layers where there were higher numbers of combjellies, suggesting a similar role like the nauplii as feeding source for the smallest *M.leidyi*. In fact even if in the first survey the copepodites had higher density in the deeper layer, the one where the number of ctenophores was lower, this reverse trend could be explained by the low density of copepodites in this first sampling, when they would have had a weak influence on the *M.leidyi* abundance. The adult copepods were analyzed according to the species. The tendency for the adults of genus *Acartia* was to have bigger populations in the water masses where the biovolume of *M.leidyi* was bigger, suggesting that they could be prey of the adult combjellies, but since the copepods densities were not statistically different in the different layers, more samples would have been required to show this possible relationship. These results may be linked with a different feeding behavior in ctenophores of different length. It is reasonable that the youngest and smallest combjellies are more specialized in creating feeding flows to capture the little preys like the nauplii, maybe also because of the small dimensions of the lobes, used instead by the bigger individuals to capture the bigger adult copepods like *Acartia sp.* (Waggett and Costello, 1999). The situation is less clear for the genus *Oithona*. These copepods, in fact, had statistically different densities in

the water masses when there was not a difference between the jelly populations (third survey) and vice versa (first and second surveys).

But the prey abundance is not the only factor influencing the distribution in the water column. Also the oceanographical factors play an important role. In fact with strong pycnoclines the populations were separated and there were differences in the densities of individuals and biovolume between layers. When the pycnocline was weaker, like in the third survey, the density of ctenophore did not present differences between layers. It can be due to mixing forces within the water column, but also to the possibility, since the reduced osmotic stress, of an active movement of the combjellies between different layers. The influence of these physical factors, as the importance of the temperature in the cilia beating velocity, is not totally understood (Greve, 1972; Esser et al., 2004)

Other than the vertical distribution, also the abundance of *Mnemiopsis leidyi* in the Aarhus Bay was analyzed and it was found that the abundance varied over the two weeks between the first and the third sampling time. The number of individuals was stable or slightly increasing from the first to the second survey, while there was a tendency to reduction both as number of individuals or biovolume in the third survey. Different factors can explain this pattern. During the samplings, an algae bloom had occurred, followed by a zooplankton bloom, but the mean temperature of the water column lowered. If the zooplankton availability can be considered as the driving factor of abundance in the first two surveys, the reduction in abundance in the third survey can be due to physical factors. A temperature lower than 7.2 °C, like the one measured during the third survey, may be enough to lower the survival and reproduction rates of *M.leidyi*, despite the plankton bloom still present at least in the deeper layer during the third survey. In the first two surveys, in fact, the average temperature of the water masses is equal or higher than 8°C. Survival of the individuals of *M.leidyi* in the third survey despite closeness to the threshold of 4 °C considered the inferior limit to the survival of this ctenophore (GESAMP, 1997; Shiganova et al., 2001) could be explained by the importance for this species of the time of exposure to a certain temperature, more than the instantaneous temperature itself (Esser et al., 2004). In this case, the reduction in density would be just the initial stage of decline of a population exposed to the lethal temperature for a too short period. Moreover, the temperature has been measured at the beginning of the coldest season, with the snow falling at the sea level. The temperature of 4°C, is close enough to make the temperature a plausible limiting factor in a near future after these samplings.

Another interesting aspect could be the relationship between the *Mnemiopsis leidyi* and other jellyplankton genera like *Cyanea* and *Beroe*, found in the samples. Very few individuals of *Cyanea* and only one of *Beroe* were caught, though, and it has been impossible to carry out any statistical analysis. A negative correlation among densities of these jellyfish and density of *M. leidyi* would be an indirect confirmation of predation and/or competition as already shown by Hosia and Titelman for *Cyanea capillata* and *Beroe gracilis* (2011, 2011b).

The second approach used in this study involves genetic analysis, became a fundamental tools to study invasions of non-native species. Genetics is useful in assessing the effective presence of species (molecular identification), outlining populations' structure, identifying prey-predator dynamics with endemic species and reconstructing the invasion pathways and the native geographical area where the first invasive specimens have come from. In the last years works on *M. leidyi* DNA have already given back important results. The presence of *M. leidyi* in North Europe was recognized starting from 2005 (see INTRODUCTION), but it is possible that the invasion began years before. According to Holsteijn (2002), an autumn bloom of *Bolinopsis infundibulum* O.F. Müller 1779 occurred in 2002, even if this species is rare in Dutch coasts. Faasse and Bayha (2006) argued the morphological similarities of *M. leidyi* and *B. infundibulum* could have led to misidentify the taxonomical recognition. As reported in Van Ginderdeuren et al. (2012), both

combjellies species have oral lobes as main diagnostic character. The difference between them is that in *M. leidyi* lobes reach the apex of the body, ending close to statocyst while in *B. infundibulum* they end on the body between the statocyst and the mouth (Van Ginderdeuren et al., 2012). However, discerning the end of the lobes on the body can be actually a problem, in particular for small individuals where the use of stereomicroscopes is needed. Furthermore tissues from jellyfish are fragile and the fixation is not effective. Degradation of the body is rapid and morphologic characteristics are lost in short time, hindering lately identification. Therefore, molecular identification is the only way to have reliable evidence to assess the presence of the species in a survey, with the advantage of using also fixated samples without the problems of the immediate identification on the field. Starting from this need of molecular endorsement to confirm the morphological identifications, in few years several markers have been designed on jellies DNA for different purposes. This effort has been particularly great for Ctenophora, coping with the lack of basilar data as the sequence of a complete genome of a model organism and the presence of few studies and few sequences in GenBank databases. The interest for *Mnemiopsis leidyi* invasion has increased the effort on the genetic analysis of this species. Important studies based on sequencing of several markers on nDNA and mtDna led to the recent publication of the whole sequence of the nuclear DNA (Ryan et al, 2011; GenBank accession number AGCP00000000.1) and mitochondrial DNA (Pett et al., 2011; GenBank accession number JF760210.1) both in 2011.

The marker used in this study for molecular identification is ITS region. This region is unusually short in jellies (Podar et al., 2001) and contains too many repetitive sequences to draw reliable results in phylum-wide phylogenetic studies. Anyway, ITS region was used in the last year as marker for molecular specific identification, being variable enough to discriminate among species closely related (Anderson and Adlard, 1994; Dawson and Jacobs, 2001; Schroth et al., 2002; Coleman and Vacquier, 2004) and it has been already proved to work for species in the phylum Ctenophora (Bayha et al., 2004) and for *Mnemiopsis leidyi* itself (Bayha, 2005; Faassee and Bayha, 2006; Fuenteset al., 2010, Van Ginderdeuren et al., 2012). Thanks to ITS, it was possible to identify 105 *Mnemiopsis leidyi*, confirming the presence of the species for the whole sampling period. The presence of 4 *M. leidyi* morphologically identified as another species and those 18 samples misidentified as *M. leidyi* but actually belonging to other species confirm the need of the genetic marker. There are also four individuals identified as *Mnemiopsis leidyi*, but with several bases mismatching with the other known sequences, anomalous sequences that headline the need for further analysis. It has to be clarified, in fact, if those four individuals can be considered a particular cluster of *M. leidyi* or if they belong to another species. The actual lack of genetic info in the databases about ctenophores claims for further surveys in order to solve these issues (Van Ginderdeuren et al., 2012). The first molecular identification carried on samples from Italian coast during this study (Binetti et al., in press) is a molecular confirmation of reports about *M. leidyi* in the Ligurian Sea (Boero and Shiganova, 2009), but it is also a further input for databases, storing in GenBank sequences from a geographical region not considered until now. This is useful to endorse BLAST queries and to compare sequences for population genetics analysis (see below). Another important gene for molecular identification, cox1, was amplified in this study. Involved in the Barcode of Life (Ratnasingham and Hebert, 2007), fully updated databases with cox1 sequences from lot of species can be a fast tool to design universal primers for easy molecular identification or taxa specific primers useful e.g. to design fluorescent probes to real-time PCR, a technology that relates variation of fluorescence of the probes at every cycle with the amount of copies of the amplified DNA. Real-time PCR gives instantaneously qualitative data about the presence of targeted DNA sequences and quantitative data about their number of copies. Designing species-specific probes it would be possible to detect in an unknown bulk sample the identity and the relative abundance of species in it. It can be useful for example in the analysis of the stomach content of fishes in order to analyze eventual prey-predator relationships among invasive and endemic species. *Mnemiopsis leidyi* is predated

by tens of fish species in its natural habitat (Mianzan et al., 1996) and it was found in stomachs of Cod *Gadus morhua* Linnaeus, 1758 in the Baltic Sea (Schaber et al., 2011). Analyzing with real-time PCR stomachs of zooplanktivorous fishes occurring where *M. leidyi* is present, it would be possible to detect species feeding on it. In this study the new primers designed for cox1 gene were tested on 4 combjellies other than *M. leidyi* and these primers turned out to be useful to discern this species from *Beroe ovata* and *Beroe cucumis*, but they worked on *Pleurobrachia pileus* and *Lovenella assimilis* DNAs. Primers designed are so not useful to real-time PCR aimed to detect only *Mnemiopsis leidyi* in the fish stomachs. Unfortunately *Bolinopsis infundibulum* had not specimens gathered in the sample collection and primers could not be tested on it. If the probes didn't work on *B. infundibulum*, it could be a fast way to recognize molecularly these two morphologically similar species.

ITS region has also been used as a successful marker for population genetics. Ghabooli et al. (2011) found 13 different alleles discerned by 7 positions in the sequence of this diploid species. Thanks to these alleles, the "two invasion pathways pattern" was assessed, with the distinction of a northern and southern cluster. A lot of sequences obtained from MEMO samples were not completed. The sequencing reactions gave for several individuals' strings with low quality in the first or last part of sequences. For those samples information are missing about diagnostic positions that would have indentified the ITS genotype. Furthermore, a lot of ambiguities were found in the positions used to discern alleles. According to Ghabooli et al., these positions were actually SNPs, with only two bases changing in each position in the different alleles. The ambiguities found in the MEMO sequences in the diagnostic positions were between the two bases expected for each SNP. This has been considered as evidence to consider these ambiguities not as a mistake in the sequencing but as the result of heterozygosis of the samples. Where at least two positions had an ambiguity, the exact combination of the alleles in that genotype was impossible to know because of the absence of a cloning step in the experiment. Just 1 individual had the complete sequence and 1 ambiguity, being the only heterozygous whose genotype could be identified with alleles C and D (after Ghabooli et al., 2011). Furthermore a new allele was discovered in this study. It was found on two individuals, evidently homozygous for that. Also the four individuals with the anomalous sequences had other bases in those positions and, if it was confirmed they are *M. leidyi*, it would represent a 15^{th} allele. Both these new alleles have in diagnostic position 574 a new base (G) more than in the Ghabooli's study (A and T), while the allele of the anomalous samples have also in diagnostic position 212 a G not found before (only A and T). More replicates are required to see if these are sequencing mistakes or effectively new alleles. The impossibility to genotype every individual made impossible any population structure analysis out of this data.

Other kind of marker that can be used for population genetics are SNPs and microsatellites. Authors debated in the last years about the pro and cons of these two molecular markers. SNPs tests are based on differences of bases in a position in the sequences, while microsatellites are tested on the different length of their alleles and this makes SNPs less "lab-dependent" and more suitable to work on degraded DNA (Mesnick et al., 2011). SNPs are considered nowadays the best choice because they are abundant in genome and have accurate and efficient genotyping methods making easy comparing data. Moreover, they respect the simple mutation model avoiding corrections in the use of traditional divergence-based statistical method like Fst (Meirmans and Hedrick, 2010). SNPs are also bi-allelic (Chakraborty et al., 1999; Krawczak, 1999) and if this can make easier the statistic analysis, on the other hand it makes the single mutation weak from a biological point of view. A lot more SNPs need to be analyzed to screen a population in order to have the same information that can be acquired with a single microsatellite.

In this study, both SNPs and microsatellites were used. Microsatellites were used because of the great importance that the study of Reusch et al. (2010) had had about the definition of the different native geographical ranges of the two *M. leidyi* population clusters in North and South Europe with the use of

seven microsatellites. Because Reusch et al. did not consider the population from the Belgian, French and Dutch coasts a screening of the samples was carried out with primers for these seven markers and data were successfully obtained. However, a big issue affected the statistical analysis. The problem was working with samples caught from several institutes not coordinated with a uniform and planned sampling campaign, but rather dealing with several samples caught in different areas at different times, often lacking information about the geographical position of catching. The heterogeneity among groups in number of individuals, catching methods and geographical position made statistical results weak and biological meaning difficult to be found, in particular for 3 groups including just 1 individual. A total of 10 populations were identified out of the 69 genotyped samples according to date of sampling and institute that had carried out the survey. Average number of alleles over the seven markers was positively correlated with the number of samples in the population itself. Gene diversity, that measures the diversity based on sample size, allelic frequencies and observed number of heterozygotes, was used to calculate the diversity among locus and the L45.1 showed the highest average diversity among populations, being also the locus with highest number of alleles. Population 8 was instead the most different among groups, but having just 3 individuals requires more individuals to adjust the allelic frequencies and the actual heterozygosis. The population 9 was the one including Italian samples, but as populations 1 and 5 there was just 1 individual that could be genotyped. The test for the Hardy-Weinberg on the populations with more individuals showed for 4 groups out of 7 a divergence from the equilibrium, phenomenon that can be explained with inbreeding within the populations, as subgroups with different allelic frequencies breed together (excess of homozygosis, Wahlund effect). Another possible cause could be the selection over loci that could have increased the fitness for particular alleles or for heterozygous individuals. Also a human bias could have occurred in the analysis. The peaks in the graph to analyze the microsatellites were not too strong and also disturbed by noise. It could have made sometimes difficult to score the right peaks, in particular for some loci as L12 and L15, while in other cases two profiles of two alleles similar in length could have been scored as a single one, misleading the analyses and creating null alleles. All these bias could have affected also the calculation of F-statistic parameters locus by locus, being L13 the only locus in HW equilibrium. The significant value of Fst over all loci suggested the presence of subclusters among samples. According to the genetic distance visualized also in a 2D AFC plot, the Italian individual (group 9) seemed to be particularly different from the others. This individual could not be assigned to the population of North Sea where all the other samples came from, an indirect clue showing the difference among samples from the southern and northern clusters found in previous studies (Ghabooli et al. 2010; Reusch et al., 2010). Having just one sample and being impossible to calculate allelic frequencies and allelic richness, this result must be taken into account really carefully. Furthermore this population was taken out from analyses of pairwise Fst, MDS and of the software STUCTURE. These last 3 tests were carried out with “microsatellites populations” number 2,3,4,6,7,8 and 10, not considering the 3 populations with just 1 sample. MDS plot based on pairwise Fst table and STUCTURE bar plot showed both the presence of two subclasters with population 8 and 10 different from all the others. These two groups include the last samples arrived in the lab, but the little time gap with the groups of the other subcluster made the temporal factor not a reliable explanation for this difference. The absence of exact geographical reference for each catch heavily hampered the research of other possible explanations. This shows how important it is to gather all the data during and after the sampling and share them adequately, in particular in projects involving more institutes. To understand the reason of the presence of these subclasters more surveys are needed to be done in that area during the late autumn-first winter period.

The other part of the study was the identification of SNPs in targeted regions. For this purpose two regions of the mtDNA were taken into account, the genes cox1 and cytb. In both of them SNPs were identified for a total number of 6. These are SNPs adhering to the parameters of MAF (Minor Allele Frequency) 5% and

coverage 70%. It is important to underline also the presence of a candidate SNP in the cytb gene in position 9204. In this case MAF is under the threshold of 5%, but the samples bringing the base G instead of A are only Italian ones. It is therefore a clue about a possible diagnostic SNP. A correct and broad sampling campaign could gather samples all over the invaded range to see if this candidate SNP could be confirmed and in case used as a mean to identify samples coming from Italy, Mediterranean or the whole Southern cluster. In both genes there were positions where lots of sequences had the same ambiguity. Considering that the mtDNA is aploid, with a cloning experiment it could be possible to genotype the different copies of mtDNA that coexist in the same individual that would in this way be heterozygous.

Standing the results obtained in this study, it has to be headlined that more researches are needed to better explain the distribution and the abundance of *Mnemiopsis leidyi*. In particular more classes of zooplankton can be taken into account (Appendicularia, Echinoderm larvae, Nemertea) to better understand the food availability for this ctenophore. Furthermore, more replicates in the catching would result in more powerful statistics to reduce the variation from the values of abundance here considered, other than producing more results, for example about the relationship with *Cynea* and *Beroe*. Also better sampling campaign to obtain individuals to screen with genetic markers would turn out in more statistical powerful results with a greater biological meaning.

5 CONCLUSIONS

In this study the presence of Mnemiopsis leidyi has been proved in the two areas surveyed, the Kattegat and the North Sea. In the Aarhus Bay, where two inflows of waters are detected, the superficial water comes from the Baltic Sea and hosts a *M. leidyi* population with smaller individuals than the population hosted in the deeper water, coming from the North Sea. The distribution of this species seems to be linked with the abundance of some zooplankton taxa, in particular with the small classes such as the nauplii, and with the density of the water. The temperature seems to be also important for the abundance variation of *M.leidyi*, being a probable limiting factor at least in the colder seasons.

Understanding the actual influence of different biotic and abiotic factors on the abundance and distribution of this ctenophore would be a good way to forecast the damages that it can produce in the ecosystem of the Northern Seas and maybe to help in finding an eventual solution and hopefully preventive actions to limit invasive species, a problem that poses more and more important issues along with the climate changes and the anthropogenic impact on the oceans.

The genetics analyses carried in Belgium show how this kind of study on Ctenophora must face several problems, in particular the lack of several basic information and the small amount of sequences deposited on databases as GenBank. Nevertheless recent studies show the actual possibility to work with several markers s microsatellites and the publication of nDNA and mtDNA is an important step for future studies. Data collected are useful to assess with a molecular evidence the presence of *M. leidyi* in the geographical range, but just few clues can be inferred about population structures because of the absence of n a priori sampling plan. Nevertheless, the development of new primers to amplify new regions, the detection of new markers as SNPs and the increase of the amount of sequences in databases linked to new geographical areas are important goals of this study to be considered in the optic of setting up future experiments to improve the quality and the quantity of data obtainable from DNA studies.

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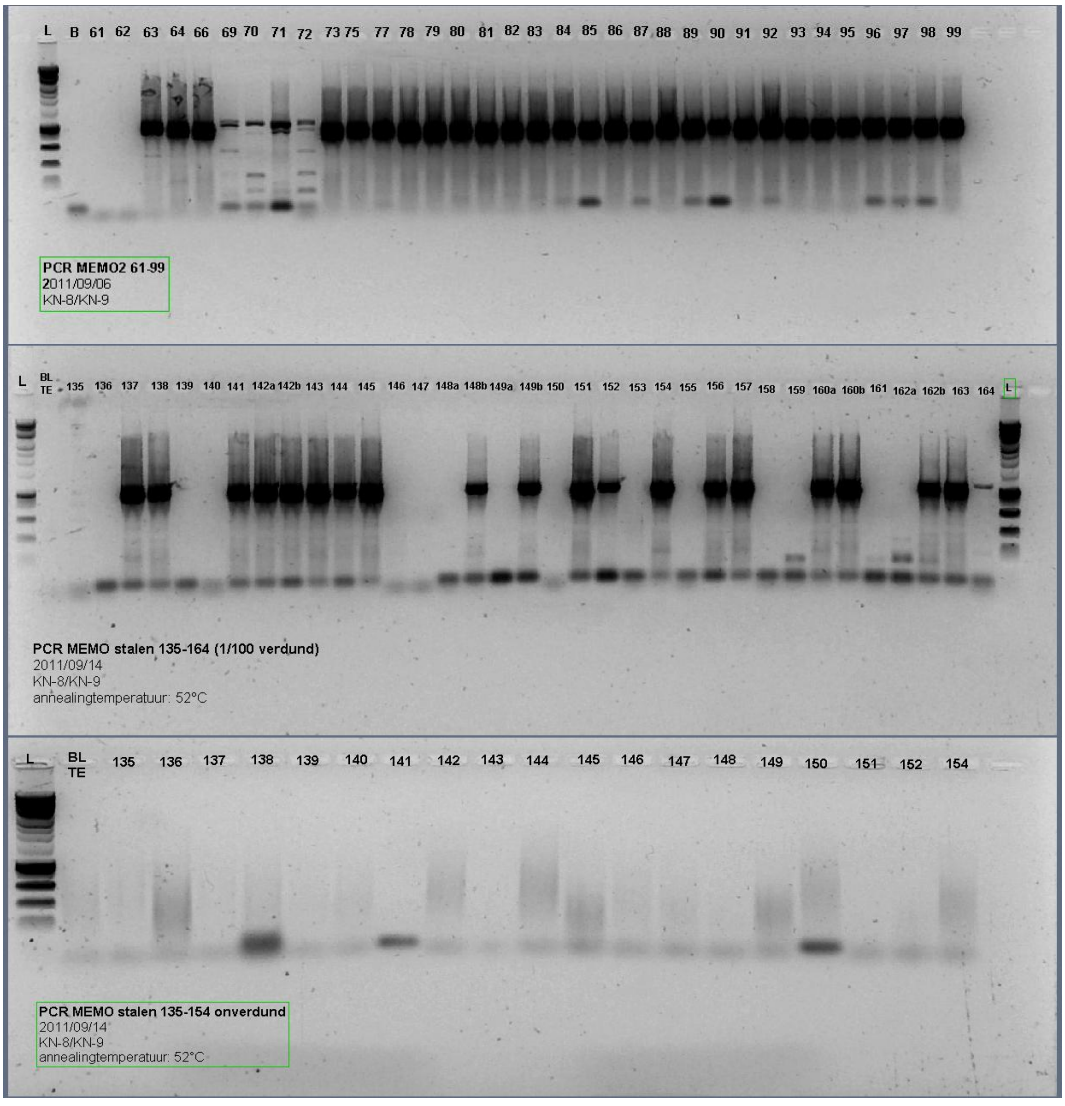
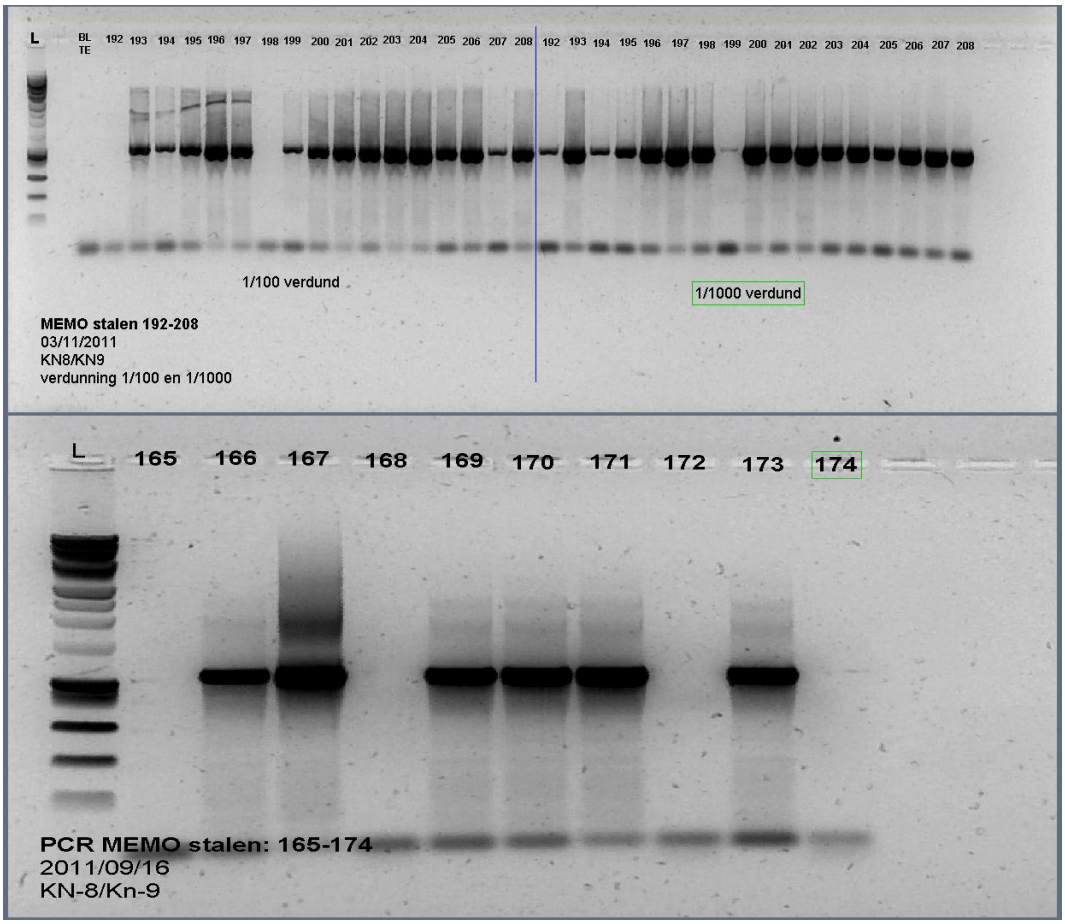
APPENDIX VI IMAGES

ITS

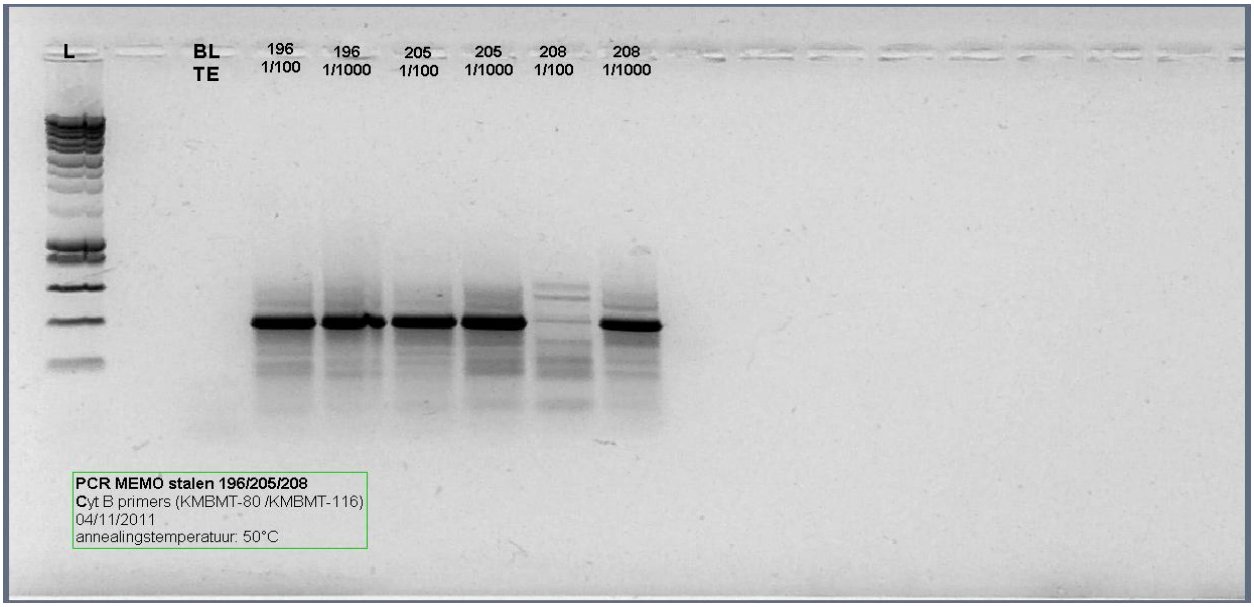
Agarose gel electrophoresis showing PCR products after amplification with primers KMBN-8 and KMBN-9 for ITS region.

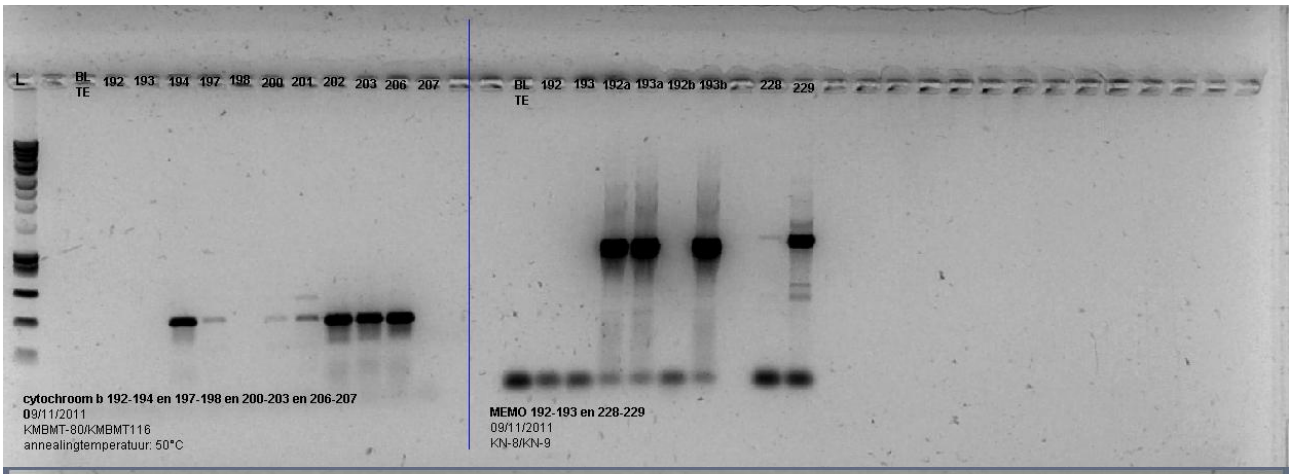




CYTB

Agarose gel electrophoresis showing PCR products after amplification with primers KMBMT-80 and KMBMT-116 for cytb region on mtDNA.

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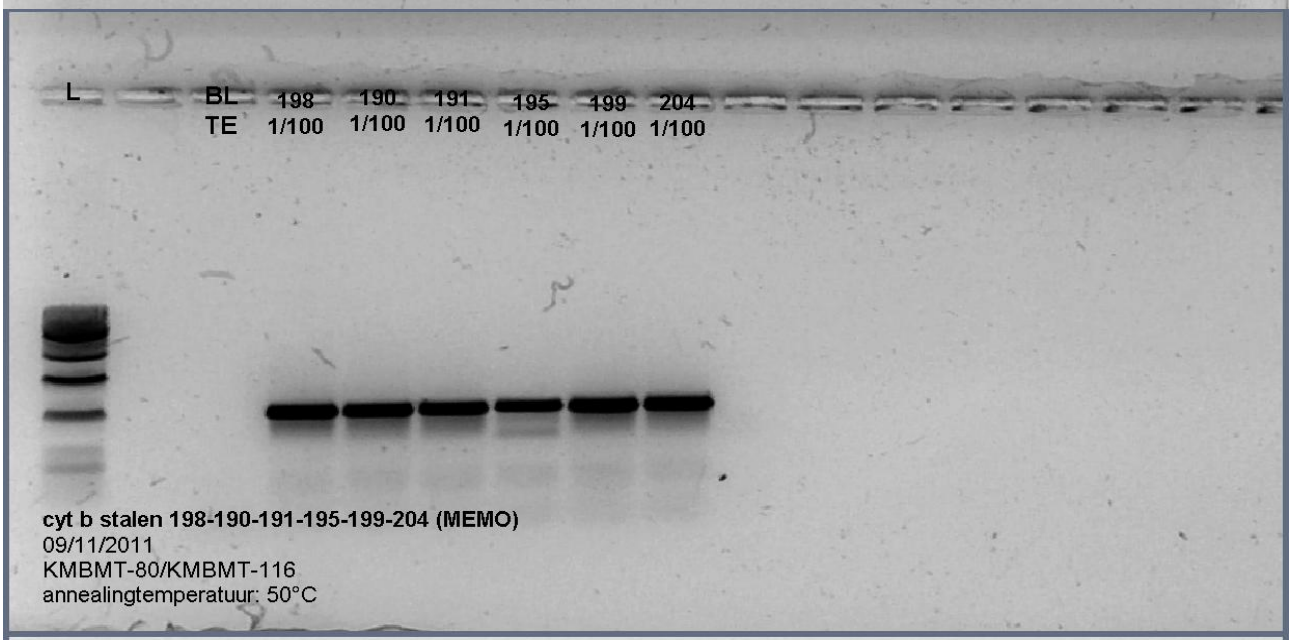
KN-8/KN-9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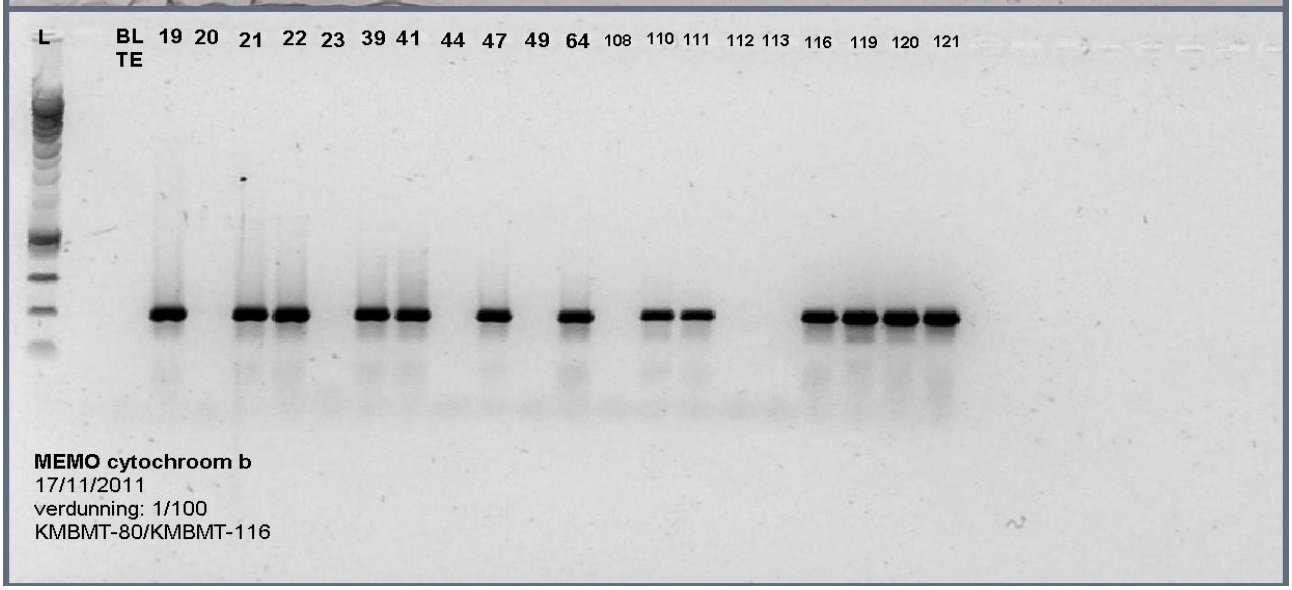
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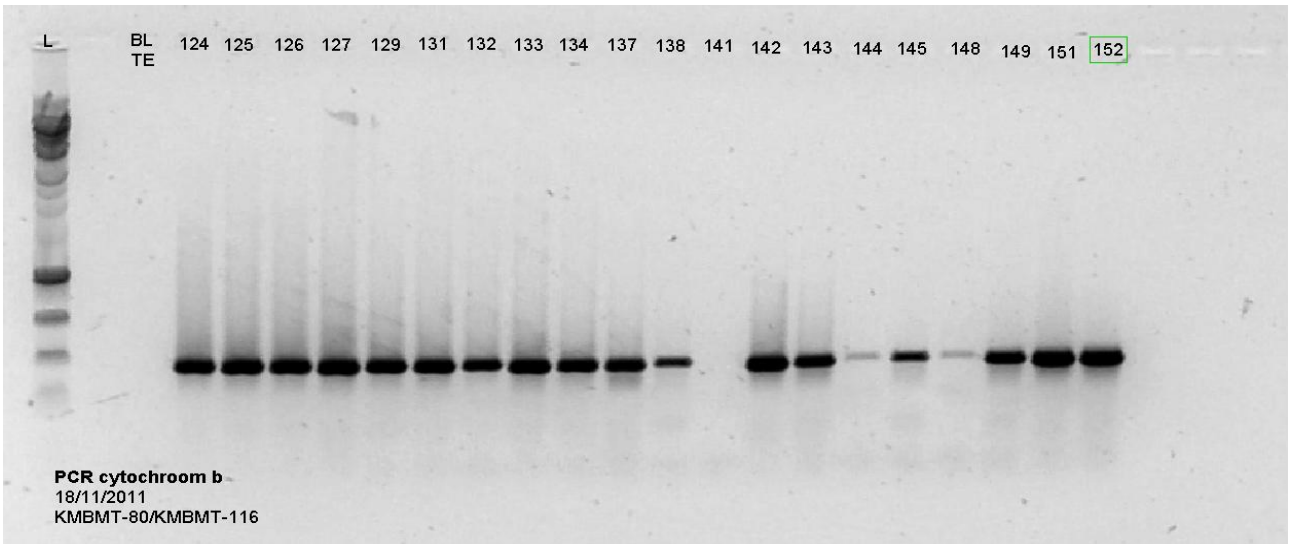
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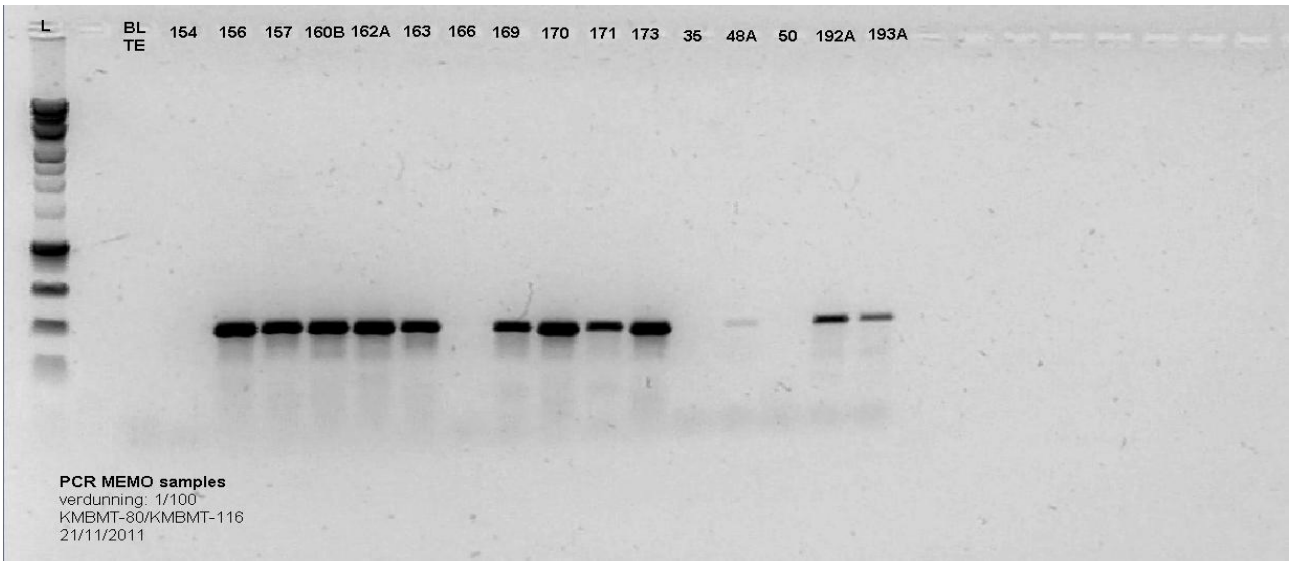
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PCR MEMO samples
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KMBMT-80/KMBMT-116
21/11/2011